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ERRATUM

Page 1981, Table II,

for NaCl g. per 10 cc. *read* NaCl g. per 100 cc.

I. THE EFFECT OF HEAT UPON THE ACTIVATING EFFICIENCY OF ENTEROKINASE.

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(Received November 6th, 1930.)

THE effect of heat upon the properties of enterokinase has not been studied in very great detail, and the work that has been done is not of a quantitative nature. Mellanby and Woolley [1913] stated that enterokinase is destroyed in 5 mins. at 65°, and is immediately inactivated in the presence of free acid. In more recent work Waldschmidt-Leitz [1924, 1925] has shown that enterokinase solutions are inactivated when heated above 50° and that they are most stable about the neutrality point. The work described in this paper deals with a quantitative investigation of the effect of heat upon enterokinase. This has been effected in principle by maintaining an enterokinase solution at a temperature of 55°, removing samples at various time intervals, and then measuring the enterokinase content of a definite volume of each sample. The procedure involves two consecutive stages.

(1) Addition of a definite volume of the solution to a constant volume of buffered trypsin solution, leaving the mixed solutions at 30° for 30 mins.¹, when formation of the compound trypsin-kinase takes place.

(2) Addition of caseinogen and buffer solution to the trypsin-kinase at the end of the above period, and then measurement of the amount of hydrolysis of the caseinogen produced in 20 mins., reckoned from the time of addition of caseinogen to the trypsin-kinase.

Before carrying out the above steps however it is necessary to define an arbitrary enterokinase unit.

The determination of enterokinase.

For the study of the effect of heat upon enterokinase it is necessary to have a method for the measurement of "quantity" of enterokinase. This measurement is arrived at by determining the activating capacity of a sample of enterokinase with respect to a known and constant quantity of trypsin. In this way both Waldschmidt-Leitz [1925] and Linderstrøm-Lang and Steenberg [1929] have defined arbitrary enterokinase units. In their determinations

¹ The diminution in the enterokinase content during this period of "activation of the trypsin" is negligible.

however the quantity of enterokinase was defined from measurements of its activating capacity with respect to dried pancreas powder or to a glycerol extract of the powder. In the present work, to avoid any possible complications from the presence of the activator in the dried gland powder, the enterokinase "quantity" has been measured by determining its activating capacity with respect to trypsin which has been purified so as to be free from enterokinase and the enterokinase pre-stage, according to the method of Waldschmidt-Leitz and Linderstrøm-Lang [1927]. Except for this modification the technique adopted for the determination of an enterokinase unit is essentially that of Linderstrøm-Lang and Steenberg [1929].

Preparations.

(a) *Enterokinase solution.* Pig's intestinal mucous membrane was treated with acetone, acetone-ether mixture and finally ether according to the procedure of Waldschmidt-Leitz [1924]. 6 g. of the dried mucosa prepared in this way were dispersed through 200 cc. of distilled water by shaking, and the dispersion was left for 24 hours in the ice chest, to allow the active material to pass into pseudo-solution. The suspension was then centrifuged and filtered. To 100 cc. of the clear filtrate were added 1.6 cc. of *N* acetic acid, and the resulting precipitate of protein was filtered off. The clear supernatant liquid containing the enterokinase was neutralised with *N* ammonia and kept in the ice chest. About 300 cc. of solution were prepared in this way and were used throughout the work described in this paper by diluting a volume of 10 cc. with 3 cc. of distilled water.

(b) *Trypsin solution.* Glycerol extract of dried pancreas powder was purified according to the method of Waldschmidt-Leitz and Linderstrøm-Lang [1927], so as to be free from enterokinase and the pre-stage of that activator. The details of this purification are given in a previous paper by the present writer [1930].

(c) *Caseinogen solution.* The caseinogen solution was prepared by adding 100 cc. of 0.05 *N* ammonia to 6 g. of caseinogen (Kahlbaum-Hammarsten) stirring and keeping for 1 hour at 30°. This solution is referred to as a 6 % caseinogen solution.

(d) *Buffer solutions.* Buffer (1) was prepared by mixing 300 cc. of *N* ammonium chloride and 50 cc. of *N* ammonia, giving a p_H of 8.3 at 30°.

Buffer (2) was prepared by mixing 250 cc. of *N* ammonia and 350 cc. of distilled water.

Procedure.

The determination of an enterokinase unit according to the method of Linderstrøm-Lang and Steenberg [1929] was then carried out as follows. 3.5 cc. of buffer (1) and 3 cc. of trypsin solution were measured into a 50 cc. Jena flask. There was then added a volume of distilled water such that after the addition of the required amount of enterokinase solution the total volume

was 9 cc. The flask with its contents was placed in the thermostat at 30° and the enterokinase solution was added at a time which was reckoned as the zero for the period of activation. (5 cc. of water, 65 cc. of 96 % alcohol and 1 cc. of a 1 % solution of thymolphthalein in alcohol were measured out into each of two 300 cc. flasks to be used for the titration of the mixture under investigation before and after the hydrolysis of the caseinogen.)

After the activation period of 30 mins., 6 cc. of buffer (2) (warmed to 30°) and 15 cc. of a 6 % caseinogen solution (also previously warmed to 30°) were added. The whole was shaken and, at a time reckoned as the zero for the period of hydrolysis, 10 cc. of the mixture were pipetted into one of the titration flasks containing alcohol. (The alcohol entirely stops the enzyme action without perceptibly precipitating the caseinogen, thus making it possible to keep the mixture for later titration.) At the end of the reaction period of 20 mins. the same 10 cc. pipette was rinsed with the liquid under investigation, and 10 cc. were pipetted into the second titration flask containing alcohol. Each titration flask contained one-third of the amounts used in the experiment. The contents of each flask were then titrated in the stepwise manner described by Willstätter *et al.* [1926]. The difference between the two titration values is equal to the number of carboxyl groups formed during the hydrolytic period of 20 mins. in 10 cc. of the experimental liquid, expressed as cc. of 0.2N NaOH.

By using various quantities of kinase solution and making measurements in this way a relation was obtained between the quantity of enterokinase and the extent of the hydrolysis of the caseinogen. This is shown in Table I.

Table I.

Volume of enterokinase solution cc.	Increase in acidity cc. 0.2N NaOH
0.05	0.53
0.10	0.80
0.20	1.04
0.30	1.12
0.50	1.19

An enterokinase unit was defined as the quantity of enterokinase which produces an increase in acidity equivalent to 1.04 cc. of 0.2N NaOH under the conditions given above. By plotting the results of Table I and using this definition of an enterokinase unit the following values, set down in Table II, were obtained for fractions and multiples of the unit.

Table II.

Enterokinase units	Corresponding increase in acidity cc. 0.2N NaOH
0.25	0.52
0.50	0.80
1.00	1.04
1.50	1.12
2.00	1.18
2.50	1.19

These results are plotted in Fig. 1, and the curve thus obtained is used for determining "quantity" of enterokinase.

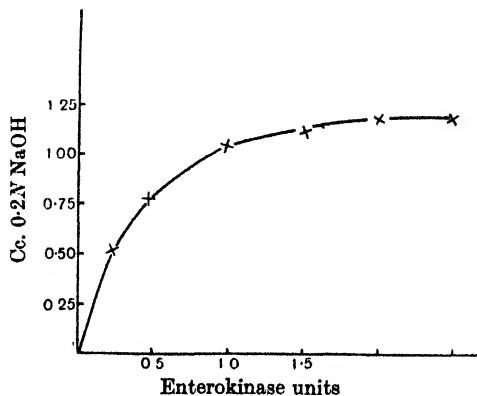


Fig. 1.

The velocity of inactivation of enterokinase by heat.

Having now established a unit of enterokinase activity, we can proceed to measure the rate of thermal inactivation.

To 10 cc. of the enterokinase solution, prepared in the manner described above, were added *N* acetic acid or *N* ammonia to bring it to a required p_H , followed by distilled water to make the total volume 11 cc. This volume of solution was measured into a number of test-tubes already immersed in a thermostat at 55°. The test-tubes were then tightly stoppered and transferred at various time intervals from the thermostat into crushed ice. The enterokinase content of 0.2 cc.¹ of solution was then measured in the manner described above. The p_H measurements throughout the work described in this paper were made at room temperature by means of the glass electrode.

In the following tables the enterokinase content is expressed in terms of the enterokinase unit defined previously and is set down as enterokinase units per cc. of solution. The readings for each time interval were made in duplicate and the enterokinase content recorded for a particular time interval is the mean of the two readings.

The course of the reaction. There is no reference in the literature to the course of the reaction when enterokinase is inactivated by heat. It was found in this work that the effect of heat upon the activating efficiency of enterokinase could be expressed fairly well by the unimolecular expression:

$$k = \frac{2.303}{t} \log_{10} \frac{a}{a-x}$$

where a is the initial concentration of enterokinase per cc., $a - x$ is the concentration of enterokinase after time t mins.

¹ This volume was chosen so as to bring the readings on the "steep" part of the curve. If too large a volume is chosen we are in the region of maximum activation and consequently there may be no apparent decrease on heating.

ENTEROKINASE

The agreement with the unimolecular expression is illustrated in Table III, which gives some idea of the degree of constancy obtained in average experiments. In some of the experiments there was a tendency for the constants, worked out on the basis of the unimolecular-law, to fall. It could not be decided whether this was a real effect or whether it was due solely to experimental error. It should be pointed out in this connection, however, that the experimental observations have not been confined to early stages of the reaction, but that they have covered about 75 % of its course.

Table III.

Temperature 55°.

	Time (mins.)	Enterokinase units per cc.	k^*
p_H 8.47	0	6.00	—
	20	3.56	2.61×10^{-2}
	40	2.12	2.59×10^{-2}
	60	1.37	2.46×10^{-2}
p_H 6.40	0	6.00	—
	45	3.50	1.20×10^{-2}
	95	2.00	1.16×10^{-2}
	143	1.25	1.10×10^{-2}

* In this table and throughout k represents the velocity constant of a unimolecular reaction.

The effect of p_H upon the inactivation of enterokinase by heat.

The p_H of the medium has a marked effect upon the stability of enzymes, and the following results, which show that the stability of enterokinase is also dependent upon p_H , emphasise how closely this activator resembles an enzyme in certain of its properties. In Table IV the results obtained for the inactivation of enterokinase by heat at various p_H values are summarised. The mean of the values obtained for k at a particular p_H are set down opposite that p_H value.

Table IV.

Temperature 55°.

p_H	$k \times 10^2$
4.00	3.29
4.56	2.67
5.12	1.30
6.40	1.15
7.83	1.30
8.47	2.53
9.01	6.06

The results of Table IV are presented graphically in Fig. 2, where it is seen that the optimum stability region for enterokinase is fairly broad and lies between p_H 6 and p_H 7.

The critical increment of the process of the inactivation of enterokinase by heat.

Experiments were carried out to determine the critical increment of the heat-inactivation of enterokinase by measuring k at 50° and at 60° for the

following three cases: (1) at the region of optimum stability, *i.e.* at p_H 6.5, (2) at p_H 5.12, (3) at the still more acid reaction of p_H 3.2.

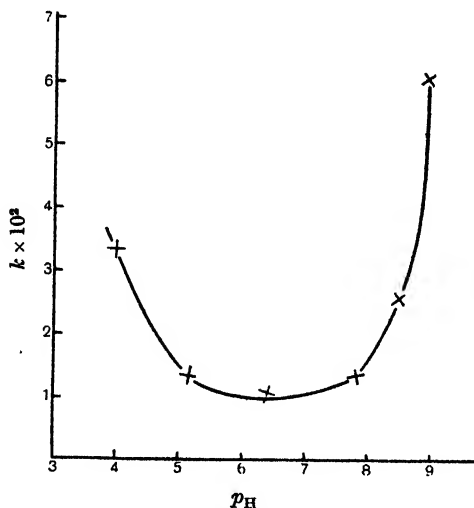


Fig. 2.

Table V.

p_H	k at 50°	k at 60°	E (cals.)
6.50	4.42×10^{-3}	3.27×10^{-2}	42,600
5.12	4.95×10^{-3}	3.52×10^{-2}	41,700
3.20	1.06×10^{-2}	8.57×10^{-2}	44,000

The results are summarised in Table V in which the figures in the last column represent the critical increments calculated by substituting the mean values of k at 50° and 60° into the integrated form of the equation:

$$\frac{d \ln k}{dT} = \frac{E}{RT^2}.$$

Thus it is seen that the critical increment for the heat inactivation of enterokinase is the same over the p_H range 3–7 and is of the order of 40,000 cal. per “molar unit” of enterokinase. This value of the critical increment is substantially the same as that obtained by the writer [1930] for the heat-inactivation of trypsin itself.

It is interesting that the results obtained with enterokinase, a specific activator and not an enzyme *per se*, show such a close resemblance to those obtained with the enzyme trypsin.

SUMMARY.

Experiments have been carried out on the determination of an enterokinase unit. The method adopted was that of Linderstrøm-Lang and Steenberg, except that trypsin purified so as to be free from enterokinase and its pre-stage was used instead of a glycerol extract of the dried pancreas gland.

In this way an enterokinase unit has been defined and employed in the work described later.

The effect of heat upon enterokinase has been studied. It was found that the course of the heat-inactivation may be described fairly well by the unimolecular expression. In this respect enterokinase resembles purified trypsin.

The effect of p_H upon the stability of enterokinase has also been investigated. It was found that the stability is influenced considerably by the p_H of the medium, but there is a fairly broad optimum region of stability lying between p_H 6 and p_H 7.

The critical increment of the process has been determined at three p_H values: 6.5, 5.12 and 3.2. It was found that the critical increment is the same at all three points and is of the order of 40,000 cal. In this respect again enterokinase exhibits a marked resemblance to purified trypsin.

The above work was carried out under the direction of Prof. W. C. M. Lewis, to whom the writer is indebted for help and advice.

The writer desires to express his thanks to the Department of Scientific and Industrial Research for a Maintenance Grant, and also to Imperial Chemical Industries, Ltd., for a grant to the Department of Physical Chemistry of the University of Liverpool.

REFERENCES.

- Linderstrøm-Lang and Steenberg (1929). *Compt. Rend. Trav. Lab. Carlsberg*, **17**, No. 16.
Mellanby and Woolley (1913). *J. Physiol.* **47**, 339.
Pace (1930). *Biochem. J.* **24**, 606.
Waldschmidt-Leitz (1924). *Z. physiol. Chem.* **132**, 181.
—— (1925). *Z. physiol. Chem.* **142**, 217.
—— and Linderstrøm-Lang (1927). *Z. physiol. Chem.* **166**, 241.
Willstätter, Waldschmidt-Leitz, Dunaíturria and Kunstner (1926). *Z. physiol. Chem.* **161**, 191.

II. ON THE QUANTITY OF FREE WATER IN THE RED BLOOD CORPUSCLES.

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(Received November 24th, 1930.)

THE quantity of free water in the red blood corpuscles is of great significance for a wide field of research, and particularly for the study of the distribution of diffusible substances between the corpuscles and plasma.

In all previous work in this field [Warburg, 1922; Van Slyke, 1926; Henderson, 1928], Ege's [1922, 1] figure of 35–40 % for the disperse phase of the corpuscle, *i.e.* 65–60 % for the free water, has been the basis of calculation. With this value directly or indirectly inserted in the formulae, it has been shown that the distribution of Cl^- and HCO_3^- is inversely proportional to the distribution of H^+ . The value of free water only enters the calculation of concentrations of the first named ions, as $[\text{H}^+]$ is measured directly as the activity of the blood cell interior. Netter [1929] in the same way has shown that the distribution of NH_4^+ and H^+ are directly proportional. $[\text{H}^+]$ here is likewise measured directly, $[\text{NH}_4^+]$ computed from the analyses with the value of free water taken as 65 %.

This is all quite in accordance with the Gibbs-Donnan theorem and forms, I think, a strong argument in favour of the above-mentioned value or the free water.

Determination of the quantity of free water in the erythrocytes can be made in several ways, all of them indirect. That most commonly used is the measurement by the hematocrit method of the volumes of the same mass of red corpuscles in several hypo- and hyper-tonic solutions of the same salt. The values found are inserted in the equation for van't Hoff-Boyle-Mariotte's law: $(v - x)p = (v_1 - x)p_1$, where v and v_1 are the volumes of the cells in solutions of osmotic pressures p and p_1 respectively; the volume of the blood in its own plasma being taken as 100, x then represents the disperse phase and $100 - x$ the free water. Now, Krevisky [1930] has determined this quantity once more and found it to be only 35 %, thereby confirming an older finding by Gough [1924]. Ege [1927] showed that the technique employed by Gough was not irreproachable, giving too high volume values and therefore apparently too small a quantity of free water.

Krevisky uses a high-speed centrifuge until constant volume is reached, but he has only applied highly hypertonic (3–20 %) NaCl solutions as exterior

fluid. In such solutions Koeppe's criterion (*i.e.* a transparency of the column in the hematocrit tube) does not appear and haemolysis very often occurs. The cause of the non-appearance of Koeppe's phenomenon is perhaps that the blood-corpuscles in such solutions assume "thorn-apple" form and therefore cannot pack so closely that all fluid between them is pressed out, this being the condition for the appearance of transparency. The volume values in such concentrated salt solutions are higher than expected according to the above equation, from the values found for v and x in hypo-, iso- and slightly hyper-tonic solutions of the same salt—even if centrifuging is carried on for long periods. Krevisky, in order to make this law valid, enlarges the value of x up to 65 %. This is only what Ege [1922, 2] already observed. The latter author found the following values with dog's blood centrifuged at 8000 r.p.m. for 3-4 hours:

NaCl %	Δ	vol.	x calculated by present author
0.91	0.56	100	—
1.67	1.00	75	43
5.0	2.95	62.4	53.6
6.7	4.95	68.2	64
10.0	5.90	67.4	64

With Ege's volume values in concentrated NaCl solutions inserted in the formula, one finds apparent x values of exactly the same magnitude as Krevisky found in the same solutions. It is not however permissible to assume the extension of the validity of these values to any other range of concentration, be it higher or lower, especially when this has not been investigated. All volume-concentration curves not exceeding about $1\frac{1}{2}$ times the isotonic concentration (Krevisky's lowest is about 3 times isotonic) yield an x value of about the same order of magnitude as Ege's (35-40 %) [*cf.* Christensen and Warburg, 1929].

Whether this discrepancy between the values of x in high and low ranges of concentration is due to a real alteration in the magnitude of x or only to the impossibility of getting correct volume values in concentrated salt solutions by the hematocrit method, I am not at present able to say.

SUMMARY.

The volume values of blood corpuscles in isotonic, hypotonic and slightly hypertonic salt solutions, when inserted in the equation for van't Hoff-Boyle-Mariotte's law, yield values of about 65 % for the quantity of free water. The same blood in highly hypertonic solutions of the same salt gives lower values for the quantity of free water—down to 35 %. The first-named value has been employed in all theoretical work up till now with good result; the value of 35 % must be either fallacious, or only valid in very concentrated solutions.

REFERENCES.

- Christensen and Warburg (1929). *Acta Med. Scan.* **70**, 286.
Ege (1922, 1). *Biochem. Z.* **130**, 99.
—— (1922, 2). *Biochem. Z.* **134**, 234.
—— (1927). *Biochem. J.* **21**, 967.
Gough (1924). *Biochem. J.* **18**, 202.
Henderson (1928). *Blood*. (New Haven.)
Krevisky (1930). *Biochem. J.* **24**, 815.
Netter (1929). *Pflüger's Arch.* **222**, 724.
Van Slyke (1926). *Factors affecting the distribution of electrolytes, water, and gases in the animal body*. (Philadelphia and London.)
Warburg (1922). *Biochem. J.* **16**, 153.

III. HYDROXYPYRUVIC ALDEHYDE: ITS PREPARATION AND PHYSIOLOGICAL BEHAVIOUR.

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(Received November 24th, 1930.)

PREPARATION OF HYDROXYPYRUVIC ALDEHYDE.

THE general methods for the preparation of α -keto-aldehydes, devised by Wohl and Lange [1908] and Meisenheimer [1912] and developed so successfully by Dakin and Dudley [1914], being inapplicable to the case of hydroxypyruvic aldehyde, it is not surprising that this compound remained unknown until 1926, when its preparation was described by Evans and Waring [1926].

The method adopted by these workers consists essentially in the oxidation of dihydroxyacetone by an excess of finely powdered copper acetate suspended in water. Oxidation is allowed to proceed for 7–10 days at room temperature, when the precipitated cuprous oxide is removed by filtration and the filtrate is freed from the excess of copper by hydrogen sulphide. The resulting "golden yellow" solution is then concentrated under diminished pressure to an oil, which when the pressure is lowered to 9 mm. forms white crystals (M.P. 99°). The yield is 80 % of the theoretical, and the results of qualitative tests and molecular weight determinations by the cryoscopic method, accord with the supposition that the white crystalline material is a trimeride of hydroxypyruvic aldehyde.

Following the details for the oxidation, as described by Evans and Waring, we have confirmed that the weight of cuprous oxide produced after a period of 8 days corresponds with 97.2 % of the theoretical amount required for the usage of one equivalent of oxygen. We doubt, however, whether it is correct to conclude from this result that hydroxypyruvic aldehyde is the sole product of the reaction, and we have failed so far to obtain the crystalline trimeride. Kermack, Lambie and Slater [1929] were also unsuccessful in obtaining a crystalline product under the conditions indicated by the American workers, but claimed that the syrup produced rapidly crystallised if the oxidation had been allowed to continue for 30 days at room temperature. With this modification, we found that the cuprous oxide was contaminated with a greyish white crystalline precipitate of copper oxalate, and, moreover, the total weight of cuprous oxide was then greater (by 19–20 %) than that required for one oxygen equivalent. Hence the resulting product was less pure and was in

fact shown to contain other oxidation products including oxalic acid. The syrupy product when the pressure was diminished to 9 mm. soon passed into a pseudocrystalline mass, which was readily detached from the distilling flask and melted indefinitely just under 100° to a clear yellow liquid. It showed no crystalline structure, and no crystallising medium could be discovered. When the dry material was warmed at 4 mm. pressure, a sublimate of anhydrous oxalic acid formed, and on further heating decomposition of the bulk of the material took place. The product obtained by Evans and Waring's method showed similar behaviour, but yielded no sublimate and was thus probably free from oxalic acid. It may be mentioned that Friedemann [1927] had employed the same method for the preparation of a solution of hydroxypyruvic aldehyde, but made no attempt to isolate the product in the solid state. He considered the oxidation complete after 7 days at room temperature. We have formed the opinion that oxalate formation begins soon after this period, and at any rate is evident after 10 days, when separation of copper oxalate occurs.

The pseudocrystalline solid, obtained as indicated above, was found to be insoluble in all the ordinary solvents except water and alcohol. In the dry state it was practically odourless, but its alcoholic or aqueous solution soon developed an offensive smell characteristic of sulphur compounds, and later sulphur was gradually and continuously deposited. Evans and his co-workers [1928], in their experiments on the oxidation of sugars with copper acetate, add sodium chloride to prevent the formation of colloidal cupric sulphide, but make no mention either of this difficulty or of the presence of sulphur compounds in their preparation of hydroxypyruvic aldehyde. Kermack, Lambie and Slater, however, state that it is difficult to free the product from hydrogen sulphide and colloidal sulphur; in our material the presence of a sulphur derivative was further indicated by the fact that the solutions gave a very marked reaction with sodium nitroprusside. In other respects our product exhibited all the reactions of hydroxypyruvic aldehyde. Both the phenylosazone and the *p*-nitrophenylosazone were obtained in good yield, although during their formation hydrogen sulphide was evolved from the reaction mixtures. The phenylosazone after crystallisation from benzene melted at $132-3^{\circ}$ and decomposed about 170° : while the crude *p*-nitrophenylosazone melted with decomposition at 234° , and after recrystallisation from nitrobenzene decomposed sharply at 292° , and had N 23.02 % ($C_{15}H_{14}O_5N_6$ requires N 23.46 %). Evans and Waring quote $230-2^{\circ}$ as the melting-point of this compound, but this value is evidently too low, as Dakin and Dudley [1913] describe the compound as decomposing with the evolution of much gas at 315° . Further, it is to be noted that the formation of these osazones is no proof of the presence of the keto-aldehyde in the product, since the same osazones would arise from dihydroxyacetone. However, we found (1) that the product from the oxidation forms the osazones much more readily than does dihydroxyacetone, and (2) that the crop which separates after 4 hours'

interaction between *p*-nitrophenylhydrazine and dihydroxyacetone has a lower melting-point (243°) than the second crop separating after 36 hours (257°). This is probably due to the presence of varying amounts of hydrazone along with the osazone, as the crop melting at 243° had N 21.04 %, a value intermediate between those of the hydrazone (18.67 %) and osazone (23.46 %).

As a freshly prepared aqueous solution of the pseudocrystalline material gave no immediate Schiff reaction, whereas a solution previously boiled did so at once, the product was evidently present in a polymerised form as suggested by Evans and Waring. The molecular weight determinations quoted by these workers, however, are rendered somewhat unreliable owing to the presence of by-products containing sulphur. The exact nature of these by-products has not been determined so far, but it may be recalled that aldehydes in acid solution on treatment with hydrogen sulphide give rise to complexes containing the corresponding thioaldehydes, which on oxidation yield sulphones, a class of compounds with well recognised hypnotic properties. Accordingly, compounds of this character may here be involved, and we have invariably found it impossible, with the hydrogen sulphide method, to obtain a product, which gives no nitroprusside reaction. The main product of the action of copper acetate on dihydroxyacetone is probably hydroxypyruvic aldehyde, but while the excess of copper is most conveniently removed by means of hydrogen sulphide, the use of the latter introduces a complication.

Being unsuccessful with Evans and Waring's method and being in need of a reliably pure specimen of hydroxypyruvic aldehyde in order to test its physiological action, we have tried other methods of preparation. Thus the phenylosazone was prepared from dihydroxyacetone and experiments were carried out to transform it into the osone (1) by Fischer's method with fuming hydrochloric acid, and (2) by the benzaldehyde method. Unfortunately neither process proved successful; in the former extensive decomposition occurred, and the osazone proved too insoluble in water for the latter method to be applicable.

An experiment in which copper acetate was replaced by the corresponding silver salt led to a curious result. After allowing the oxidation to proceed, and subsequently removing the excess of silver by addition of dilute hydrochloric acid, there was obtained on evaporating the solution *in vacuo* a white amorphous product, resembling starch in appearance. It was free from inorganic material, but had no definite melting-point. Once isolated it was sparingly soluble in all solvents, but an aqueous suspension of the material reduced Fehling's solution vigorously in the cold. The sparing solubility of the product precluded the testing of its physiological action, but it was considered to be either a highly polymerised form of hydroxypyruvic aldehyde, or a condensation product analogous to that described by Levene and Walti [1928]. The material has so far not been further examined, but, as a second experiment yielded a somewhat similar result, its preparation is being repeated on a larger scale.

Attempts were then made to modify the copper acetate method of Evans and Waring, avoiding the use of hydrogen sulphide. The oxidation was carried out exactly as described by the American workers, and various methods were then tried for the removal of the excess of copper from the green-coloured filtrate. The only process which proved successful was the careful addition, with cooling and stirring, of saturated baryta until the acid reaction to litmus disappeared. The bulky precipitate containing the cupric hydroxide settled on standing overnight, and the colourless filtrate was freed from barium by means of sulphuric acid, excess of acid being carefully avoided. The filtered solution was then concentrated *in vacuo* to a syrup. This was extracted with alcohol and the filtered alcoholic extract was again evaporated under diminished pressure to a syrup, which under 6–9 mm. passed into the pseudo-crystalline state. The product obtained in this way had all the properties of a polymeride of hydroxypyruvic aldehyde. An unheated solution gave no immediate reaction with Schiff's reagent, but a solution which had been heated for a few minutes and subsequently cooled gave a marked reaction at once: it also reduced Fehling's solution in the cold and gave an intense coloration with phosphotungstate, but a negative test with sodium nitroprusside. It gave a purplish-red coloration with the phenol reagent used by Kermack, Lambie and Slater [1926] for the detection and estimation of dihydroxyacetone, and readily formed a *p*-nitrophenylosazone, m.p. 285°. No attempt was made to estimate by molecular weight determinations the degree of polymerisation, as this will probably vary in different experiments. The material was not definitely crystalline, but was undoubtedly hydroxypyruvic aldehyde, and not being contaminated with sulphur derivatives was purer than that previously described. In view of the ease with which keto-aldehydes are affected by alkali, a control experiment was carried out, in which a mixture of glyoxal and copper acetate was subjected to the same baryta treatment as outlined above. The glyoxal was quite unaffected by the process.

PHYSIOLOGICAL ACTION OF HYDROXYPYRUVIC ALDEHYDE.

As already reported [Hynd, 1930], the toxic action on mice of hydroxypyruvic aldehyde prepared by the method of Evans and Waring was observed by me 3 years ago, but the matter was not further investigated until Kermack, Lambie and Slater [1929] published their paper claiming that the dimeric form of hydroxypyruvic aldehyde produced symptoms similar to those following the administration of glucosone.

The material obtained by the methods described above has been injected subcutaneously into normal mice, and the effects produced have been carefully studied in a large number of experiments. Some typical cases are detailed in Table I, in which are also included experiments in which charcoal was employed to remove colloidal copper sulphide from the filtrate after the treatment with hydrogen sulphide. As we were concerned mainly with the effect of hydroxypyruvic aldehyde prepared by different processes, the con-

centration of the solutions used for injection was in all cases 15 %, and a uniform dose of 0.05 g. per 20 g. mouse was employed.

Table I. *Effect of subcutaneous injections into normal mice of hydroxypyruvic aldehyde prepared by different methods.*

Method of preparation	Test with nitro-prusside	Behaviour of animal	General inference
I. Copper removed by hydrogen sulphide	+	1. Became inactive almost at once. After 1 minute legs sprawling; then began to gasp. Rolled over on its side, and lay comatose. Two minutes after injection, animal suddenly righted itself: then followed general convulsive movements. Dead in 3 minutes after injection	The material has a very rapid effect, and is highly toxic
Do.	+	2. Breathing difficult after 1 minute. After 2 minutes convulsion, followed by slight recovery, when animal walked about in an excited condition. A second convulsion after 4 minutes, then coma and death in 6 minutes	Do.
Do.	+	3. After 2 minutes very irritable, sprawling limbs, rapid breathing. Jumps if handled. Convulsion in 4 minutes: dead 5 minutes after injection	Do
II. Copper removed by hydrogen sulphide and the filtrate shaken with charcoal	+	1. Slight sprawling in 2 minutes. Rapid respiration. Disinclined to walk unless disturbed. Then became very drowsy and remained in that state all day. Found dead next morning	Effect is slow: drowsiness the main symptom
Do.	+	2. Tail erect: very irritable for 5 to 10 minutes after injection. Then gradually became lethargic, and died in 6½ hours	Do.
Do.	+	3. Active for 2 minutes after injection. Then sat quietly. Irritable when disturbed. Very drowsy after 20 minutes. Slight convulsion after 1 hour. Then moribund: died after 3 hours	Do.
III. Copper removed by means of baryta	-	1. No effect was noted during first hour after injection. Then gradually became drowsy and remained so all day. Refused to take food. Normal next day	Slow effect. Drowsiness the main symptom
Do.	-	2. No symptoms except drowsiness, which developed gradually during forenoon. Continued very drowsy all day. Found moribund next morning. Died 26 hours after injection	Do.
Do.	-	3. Slight sprawling 2 minutes after injection. Rapid respirations. Then became drowsy. When disturbed moved about with difficulty. Remained in this condition all day. Normal next morning	Do.

In the above experiments, apart from the concentration of the solution at 30° under diminished pressure, no heating was employed in the course of the preparation of the material used for the injections. All the solutions used gave negative tests to Schiff's reagent. Boiling for 5 to 10 minutes gave solutions, which when cold responded to Schiff's test readily, and the effects of the injection of these solutions are shown in Table II.

Table II. *Effect of subcutaneous injections into normal mice of monomeric hydroxypyruvic aldehyde obtained by different methods.*

Method of preparation	Test with nitro-prusside	Behaviour of animal	General inference
I. Copper removed by hydrogen sulphide	+	1. Walking about 2 minutes after injection. Sprawling after $\frac{1}{4}$ hour. Then very drowsy and remained so until death in 5 $\frac{1}{2}$ hours	Main symptom is drowsiness
Do.	+	2. No definite symptoms developed. Animal remained quiet and drowsy all day. Found dead next morning	Do.
II. Copper removed by baryta	-	1. Slight sprawling after $\frac{1}{4}$ hour. Then very drowsy; but jumps when handled. Found dead next morning	Drowsiness the main symptom
Do.	-	2. Very little effect: tail stiff; then slight sprawling after $\frac{1}{4}$ hour. Drowsy but irritable if disturbed. Normal next morning	Do.

It should also be noted that 30 animals have been injected with hydroxypyruvic aldehyde, prepared by a method involving the use of hydrogen sulphide, and death, within 12 hours, has resulted in every case. On the other hand, of the 15 mice injected with hydroxypyruvic aldehyde prepared by the "baryta" method, 10 recovered completely, and the remainder died after periods ranging from 6 to 48 hours.

DISCUSSION.

Kermack, Lambie and Slater [1929] reported that the monomeric form of hydroxypyruvic aldehyde, although holding the aldehyde group in a more reactive condition, was much less toxic than the "dimeric" form, but furnished no explanation of this somewhat unexpected finding. From the data tabulated above, our results are seen to be in agreement with their observation that the monomeric form of hydroxypyruvic aldehyde does not produce a train of symptoms suggestive of insulin hypoglycaemia. It is also clear that the hydroxypyruvic aldehyde prepared in absence of hydrogen sulphide shows little if any difference when used in the monomeric or polymeric state. Similarly it was found that both monomeric and polymeric glyoxal exert practically the same effect when injected subcutaneously into mice. Moreover, the symptoms produced by glyoxal resemble in every detail those described for sulphur-free hydroxypyruvic aldehyde. Thus, even when a lethal dose is

administered, the onset of symptoms is not rapid: but the animal gradually becomes inactive and drowsy, and when disturbed moves about with difficulty owing to weakness of the limbs. Breathing gradually becomes more shallow and death follows, without convulsions, in 2-6 hours. This is typical of five experiments we have carried out using glyoxal purchased from the British Drug Houses.

We conclude therefore that the very rapid and highly toxic effect observed by Kermack, Lambie and Slater, which we also find to ensue as the result of injecting polymeric hydroxypyruvic aldehyde prepared by Evans and Waring's method, is not due to a difference in molecular complexity of the material used, but rather to the presence of an unstable by-product. This is supported by the fact that treatment with charcoal reduces the toxicity of the product in the same way as heating the solution. Yet, it is to be noticed, the charcoal-treated material gives no Schiff reaction, and hence contains the hydroxypyruvic aldehyde in a polymeric form.

As already indicated, we have failed so far to elucidate the precise nature of the by-product. The impurity which gives rise to the reaction with sodium nitroprusside is not alone responsible for the rapid toxic effect, as this reaction is still marked after charcoal treatment and also after heating. Yet the toxicity is greatly diminished by these procedures. Neither is the toxicity to be explained by the presence of oxalic acid, as we have shown by control experiments that rats withstand much larger doses of this acid than could possibly have been present in the solution of hydroxypyruvic aldehyde injected. Moreover, heating the solution would not materially influence the effect of an injection of oxalic acid.

Whatever the toxic material may prove to be, it is certainly highly unstable. The symptoms which follow on a subcutaneous injection are quite different from those observed after an injection of glucosone and are more suggestive of the effects produced by hydrogen sulphide or colloidal sulphur. Kermack, Lambie and Slater admit that in the preparation of hydroxypyruvic aldehyde it is very difficult to eliminate the last traces of hydrogen sulphide and colloidal sulphur, but conclude that hydrogen sulphide plays no part in the production of the toxic symptoms. From the results of several control experiments with various solutions containing hydrogen sulphide, we are quite satisfied that the amount of preformed hydrogen sulphide in the unheated solution of hydroxypyruvic aldehyde injected would give rise to no toxic symptoms. It is to be remembered, however, that an aqueous solution of the material at laboratory temperature continuously evolves hydrogen sulphide or some similar sulphur-containing product, and at the same time sulphur is deposited. It is conceivable that this process may be considerably accelerated by contact at 37° with the body tissues and fluids.

Again, although the material (*i.e.* sulphur) precipitated on heating the solution had, as shown by Kermack and his co-workers, no toxic effects, it does not follow that the substance from which this precipitate was produced

by heat had no toxic effect. Thus it is usually accepted that the toxicity of sulphur is due to its reduction in the organism to hydrogen sulphide, and that the extent to which the reduction occurs, and hence the toxicity, depends on the physical condition, being most intense in colloidal, less in amorphous, and least in crystalline sulphur. The degree of dispersion is thus an important factor in the effect of a preparation of colloidal sulphur [Messini, 1928]. Further, as is well known, the injection of colloidal sulphur gives rise to a hypoglycaemia and hence to symptoms resembling those following the administration of insulin. It has been pointed out, however, that there is a marked difference between the action of insulin and that of colloidal sulphur, for in the latter case there is no strict proportionality between the amount of colloidal sulphur injected and the intensity of the resulting hypoglycaemia [Pennetti, 1928]. Moreover, the hypoglycaemia is more prolonged and is not relieved by glucose, as it is accompanied by a toxic effect comparable with that caused by asphyxiating substances, such as hydrogen sulphide, or cyanides, on the respiratory centre.

While it seems improbable that colloidal sulphur would withstand the process of drying under a pressure of 9 mm. employed in the preparation of hydroxypyruvic aldehyde, a sulphur-containing by-product having an action similar to that indicated above for colloidal sulphur might conceivably still be present. This view seems to fit in best with the results of further experiments which we have carried out. Thus we have found that similar toxic compounds arise when solutions of dihydroxyacetone and glyoxal are subjected to the action of hydrogen sulphide, but apparently no such compounds form with either the sugars, glucose and fructose, or with glucosone.

The toxic product obtained from glyoxal and hydrogen sulphide differs from that encountered in the preparation of hydroxypyruvic aldehyde in that it is more stable to heat. Boiling a solution of glyoxal, which has been subjected to the action of hydrogen sulphide, gives rise to no turbidity, and death with convulsions results in 3-8 minutes after the injection of an amount of the heated solution containing the equivalent of a dose of glyoxal, which causes death in 2-6 hours. The same dose of the unheated solution of glyoxal treated with hydrogen sulphide causes death in 2-5 minutes.

As the sugars do not give rise to toxic products with hydrogen sulphide, the positive result with dihydroxyacetone may be due to the presence of traces of pyruvic aldehyde in the sample used. The negative result with glucosone is surprising, but it may be that the unchanged glucosone exerts a protective action against the toxic product, just as it antagonises the action of cyanides [Hynd, 1927], which it may be added, give rise to symptoms remarkably similar to those ascribed by Kermack, Lambie and Slater to the "dimeric" form of hydroxypyruvic aldehyde.

The contention of the above-named authors that hydroxypyruvic aldehyde produces symptoms similar to those caused by glucosone is absolutely refuted by the results recorded in the present communication.

SUMMARY.

1. When hydrogen sulphide is used in the preparation of hydroxypyruvic aldehyde, the resulting material is contaminated with sulphur-containing by-products. Some of these are stable to heat, others unstable, and the latter are highly poisonous, producing symptoms similar to those following the administration of cyanides.

2. Somewhat similar toxic compounds arise when dihydroxyacetone and glyoxal are treated with hydrogen sulphide, but apparently not when the sugars and glucosone are so treated.

3. A modification of the method of preparing hydroxypyruvic aldehyde is described whereby a product is obtained free from sulphur-containing compounds.

4. It is also clearly shown that no material difference exists between the effects produced by monomeric and polymeric hydroxypyruvic aldehyde, when injected subcutaneously into mice. The effect of either is practically identical with that which ensues after the injection of glyoxal, the main symptom being drowsiness.

5. The presence of an unstable, highly toxic by-product, which arises through the use of hydrogen sulphide, adequately explains the reported greater toxicity of the "dimeric" form of hydroxypyruvic aldehyde.

6. The glucosone effect is quite distinct from that produced by any of the compounds dealt with in this paper.

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REFERENCES.

- Dakin and Dudley (1913). *J. Biol. Chem.* **15**, 127.
— — (1914). *J. Biol. Chem.* **18**, 29.
Evans and Waring (1926). *J. Amer. Chem. Soc.* **48**, 2678.
— Nicoll, Strause and Waring (1928). *J. Amer. Chem. Soc.* **50**, 2267.
Friedemann (1927). *J. Biol. Chem.* **73**, 331.
Hynd (1927). *Biochem. J.* **21**, 1094.
— (1930). *J. Physiol.* **70**, *Proceedings*, ix.
Kermack, Lambie and Slater (1926). *Biochem. J.* **20**, 486.
— — — (1929). *Biochem. J.* **23**, 410.
Levene and Walti (1928). *J. Biol. Chem.* **78**, 23.
Meisenheimer (1912). *Ber. deutsch. chem. Ges.* **45**, 2635.
Messini (1928). *Arch. exp. Path. Pharm.* **127**, 366.
Pennetti (1928). *Physiol. Abstr.* **13**, 458.
Wohl and Lange (1908). *Ber. deutsch. chem. Ges.* **41**, 3612.

IV. SPECTROGRAPHIC DATA CONCERNING VITAMIN A AND LIVER OILS.

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THE object of the present work is to place on record a number of spectroscopic observations on liver oils and concentrates. The data are in the first place concerned with the more precise description of tests for vitamin A, in particular with the ultra-violet absorption and the blue colour test. In the second place, we have utilised spectrographic methods for a more penetrating investigation of the wider problem of the constituents of liver oils and concentrates. We hoped in this way to gain some insight into the sequence of changes culminating in the synthesis of fat-soluble vitamins or provitamins in Nature. Although it is too early to estimate to what extent this hope has been realised, a completely fresh aspect of the chemistry of vitamin-bearing oils emerges from the discovery of new and highly characteristic absorption spectra, which cannot be identified with the properties of the major constituents of liver oils.

It has already been shown [Morton and Heilbron, 1928; Drummond and Morton, 1929] that vitamin A is characterised by a broad, intense absorption band with a maximum near 328μ . Further experience on a wide range of liver oils and concentrates serves to confirm this view, whilst independent support is given in the work of Moore [1930], Capper [1930] and Drummond, Ahmad and Morton [1930] on the conversion of carotene into vitamin A *in vivo*.

The number of samples of crude and refined oils and of concentrates which have been examined in these laboratories is now considerable, and although the correlation between the ultra-violet absorption, the antimony trichloride colour test and the vitamin A potency is more firmly established than ever, attention must be drawn to the fact that disturbing factors can modify the results to some extent. Liver oils vary very widely and the spectroscopic tests are not immune from interference. Due allowance must therefore be made for disturbances caused by unusual composition of certain oils.

In collating the data on cod-liver oils the following facts emerge.

Liver oils. (1) The ultra-violet absorption of oils either in thin films or in alcoholic solution is characterised by a single well-defined maximum at $320\text{--}330\mu$. There is some evidence of a slight step-out in the curve near $300\text{--}310\mu$ and in a few oils of very low vitamin A potency narrow bands

between 300 and 350 $\mu\mu$ are faintly shown. A careful examination of the whole of the data, however, satisfies us that the true vitamin A ultra-violet absorption band is in a high degree continuous and free from fine structure.

The vitamin A potency as measured by absorption varies over a somewhat wide range. For a 1 cm. layer of a 1 % solution in alcohol E ($\log I_0/I$, where I_0 = intensity of incident light, I = intensity of emergent light) varies for "genuine refined" cod-liver oils from 0.2 to 2.0. The majority of the oils lie within the limits 0.5 to 1.6. (Carr and Price units are approx. 12 times the above.)

A small fraction of the observed absorption at 328 $\mu\mu$ is not due to vitamin A, but to other constituents of the oils. In order therefore for the spectrographic method to be used as a quantitative measure of vitamin A potency, a correction must be applied. For pure weakly coloured cod-liver oils it is generally satisfactory to subtract 0.2–0.3, whilst highly coloured oils of the cattle feeding variety require a somewhat larger correction.

(2) The persistence (*i.e.* $E_{\max} - E_{\min}$) of the ultra-violet band is variable. This undoubtedly arises from the fact that some fairly absorbent substance other than vitamin A may be present in genuine cod-liver oils, the proportion being liable to considerable variations. The ultra-violet absorption spectrum of any liver oil can therefore be regarded as fixing an upper limit to its vitamin A potency.

(3) Nearly every cod-liver oil exhibits selective absorption in the region 260–295 $\mu\mu$. Occasionally it has been possible to record some degree of resolution in this region but for the vast majority of cases only an inflexion near 280 $\mu\mu$ can be obtained. No clear parallelism between the absorption at 280 $\mu\mu$ and vitamin D potency has been traced, nor has it been possible to associate the absorption with the ergosterol content.

(4) With a few samples of fish-liver oils other than cod-liver oil the absorption at 328 $\mu\mu$ is very high but the band appears only as an inflexion. The band is partially masked by an intense abnormal ultra-violet absorption of unknown origin. If however the vitamin A is destroyed by irradiation or aeration and the absorption spectrum is redetermined it is found that the new curve is consistent with the removal of a substance exhibiting the normal vitamin A band.

(5) Spectroscopic examination of the blue solutions with antimony trichloride discloses a maximum which is normally within the limits 604–608 $\mu\mu$. Those samples of cod-liver oil which show the 328 $\mu\mu$ ultra-violet band clearly and with high persistence also give a blue colour characterised by a single sharp band at 604–608 $\mu\mu$ and little or no selective absorption elsewhere in the visible. This class comprises the majority of pale medicinal cod-liver oils and the parallelism between blue colour and ultra-violet absorption is well marked without the introduction of correction factors. With crude cod-liver oils of high potency it is frequently found that the blue solutions exhibit additional selective absorption in the region 565–585 $\mu\mu$. Normally this band

appears as a secondary maximum of smaller intensity than the band at $604\text{--}608\mu\mu$. In one or two samples of cod-liver oil and in a few samples of shark-liver oil the band in the region $565\text{--}585\mu\mu$ definitely predominates over the normal band. Even in such cases however the $328\mu\mu$ band is shown clearly with alcoholic solutions of the original substance; its persistence is unusually low, indicating the presence of additional ultra-violet absorption possibly related to the abnormal colour reaction referred to above.

Further work will be necessary before a decision can be arrived at as to whether the abnormal results are due to a mere displacement of the normal band in the colour test owing to some unusual property of the oil (*e.g.* high content of unsaturated free fatty acid) or to some totally new constituent.

Concentrates. When liver oil concentrates are examined, the blue solutions are found to be characterised by a sharp band with its maximum at $620\text{--}624\mu\mu$, the intensity varying directly with the intensity of the ultra-violet band at $328\mu\mu$ in the original material. The blue solutions with many concentrates also show a less intense band with a maximum at $582\text{--}593\mu\mu$; when the quantity of concentrate used is sufficient to give very deep blue colours, additional maxima have been seen at 643 and $697\mu\mu$ with two separate samples. In the two richest concentrates (from different oils) which we have as yet studied (Carr and Price blue values 14,000 and 9,400 respectively) both the broad continuous ultra-violet band with maximum at $328\mu\mu$ and the $624\mu\mu$ blue band were clearly exhibited at roughly 1200 and 800 times the intensity shown by a typical cod-liver oil. It is noteworthy that with these concentrates no other maxima were shown.

If concentrates rich in vitamin A are diluted with an inactive oil (*e.g.* seal oil) the quantities being so adjusted that the potency of the product is of the same order as that of cod-liver oil, the absorption maximum in the colour test reverts from $620\text{--}624\mu\mu$ to the normal position for a cod-liver oil, namely $604\text{--}608\mu\mu$.

As a result of various chemical treatments which cause decomposition of the material responsible for the characteristic ultra-violet band ($328\mu\mu$) shown by fish-liver oil concentrates, we have repeatedly observed absorption maxima near 394 , 370 , 350 and $334\mu\mu$. Concomitantly, the concentrates cease to exhibit the $620\text{--}624\mu\mu$ band with antimony trichloride. The capacity to give a blue colour has not however entirely disappeared for the relatively weakly coloured solutions show a band at $583\mu\mu$. Similarly, when a sample of unsaponifiable matter from sheep-liver fat was left in contact with the air for some weeks and extracted with a small quantity of heptane, the extract no longer showed the $328\mu\mu$ band but exhibited the same maxima at 394 , 369 , 349 and $334\mu\mu$ (Fig. 1) as were shown by the treated fish-liver oil concentrates. On removal of the heptane and addition of the antimony trichloride reagent a blue solution exhibiting a single band at $583\mu\mu$ was again obtained.

At the time that the above observations were made, it was already clear from the work of Drummond and Baker [1929] and from our own experience

that there was little prospect of the direct isolation of vitamin A in a state of purity from liver oils. It was also becoming evident that while the hydrocarbon carotene was not itself vitamin A [Dulière, Morton and Drummond, 1929] it was enormously potent in promoting growth [Euler, Euler and Hellström, 1928; Moore, 1929; Collison, Hume, Smedley-MacLean and Smith, 1929]. The direct attack on vitamin A was in a position of stalemate and the indirect attack through carotene seemed promising. The new absorption bands referred to above seemed worth further study since they might throw light either on the substance from which vitamin A is made or on substances derived from the breakdown of the vitamin molecule.

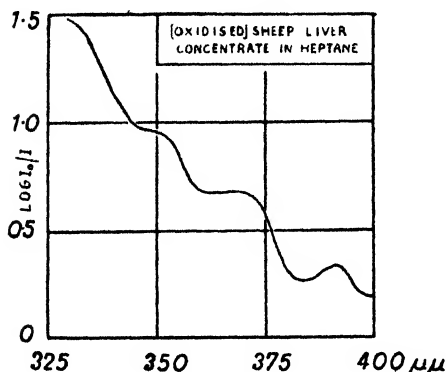


Fig. 1.

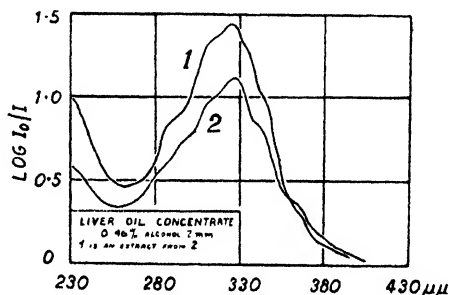


Fig. 2.

We chose first to investigate the decomposition products of the vitamin. Before however reporting on the absorption spectra of concentrates from which the typical ultra-violet band had been removed, it is necessary to reconsider this band. So far, it has been described as a broad continuous band having no recognisable fine structure.

Using a concentrate 200 times as potent as cod-liver oil we obtained indications of fine structure (Fig. 2). Attempts to increase the degree of resolution either by replacing alcohol with hexane as the solvent or by the use of still richer concentrates proved fruitless. As a result of experiments involving distribution between partially miscible solvents a very considerable increase in potency was obtained in one fraction but the indications of fine structure remained quite indefinite. As it was further found that the degree of resolution was less rather than greater, when a concentrate 1000 times as rich as cod-liver oil became available (Fig. 3) it was resolved to proceed on the two assumptions that the vitamin A band was really highly symmetrical and that the indications of fine structure arose from other substances, the relationship of which to vitamin A was unknown.

On this basis the next step was clearly to effect the removal of the $328\mu\text{m}$ band by decomposition of the vitamin in order to see if any fine-banded absorption could be revealed after eliminating the obscuring effect of the main broad band. Numerous trial experiments were made, but only one

need now be mentioned, namely the evaporation of the concentrate with strong alcoholic sodium ethoxide. An extract of this product disclosed on spectrographic examination (*a*) the absence of the $328\mu\mu$ band, (*b*) a diminution in intensity of absorption and (*c*) an increase in selectivity. With antimony trichloride a comparatively feeble blue colour was given, but the $624\mu\mu$ band was absent, the only absorption band present having its maximum at $583\mu\mu$.

As this experiment had proved fruitful, the work was repeated on a larger scale. For this experiment a neutral cod-liver oil concentrate (obtained from Messrs Lever Bros., Ltd.) having a vitamin potency of 2400 Carr and Price units was employed. As a preliminary measure, the saponification equivalent of the concentrate was determined, using a large excess of reagent and applying heat for a longer period than usual. The material was found to be practically

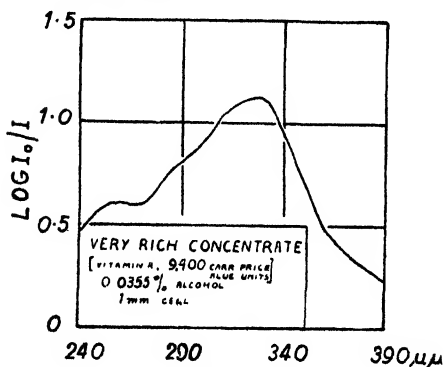


Fig. 3.

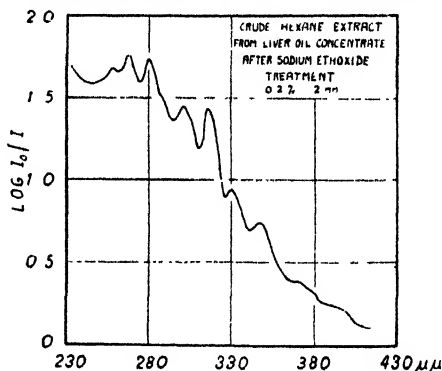


Fig. 4.

free from saponifiable matter (saponification equivalent, 19,000). A further portion of this concentrate was treated as follows. Sodium (25 g.) was dissolved in alcohol (250 cc.) and the solution was heated in an atmosphere of nitrogen in an oil-bath for 2 hours at $150\text{--}160^\circ$ with the concentrate (25 g.). The alcohol was removed under reduced pressure, the residue was dissolved in water (500 cc.) and the whole was twice extracted with ether. The ethereal solution was thoroughly washed with water, dried over sodium sulphate, and evaporated, yielding a dark, brown oil.

The colour test with antimony trichloride, however, revealed the presence of unchanged vitamin A and in consequence the above treatment was repeated. The neutral portion so obtained disclosed (as shown in Fig. 4) on spectrographic examination a series of definite maxima near 394 , 375 , 350 , 330 , 316 , 302 (inflexion 290), 282 , 271 and $260\mu\mu$. With antimony trichloride only a band at $583\mu\mu$ could be recorded. The combined alkaline liquid from the above experiments was acidified with dilute sulphuric acid and the precipitated oil was extracted with ether. The ethereal solution was shaken with dilute sodium carbonate solution to remove acids, leaving in the ether some neutral resinous material. The acids were precipitated from the sodium carbonate solution and obtained as a dark brown oil (about 2 g.). It will be noted

that the weight of acids isolated is between 5 and 6 times in excess of that demanded by the saponification equivalent (assuming saponifiable matter to be cod-liver oil glycerides) and corresponds to approximately 0.06 % of the original cod-liver oil.

The absorption spectrum of these acids is reproduced in Fig. 5; it will be

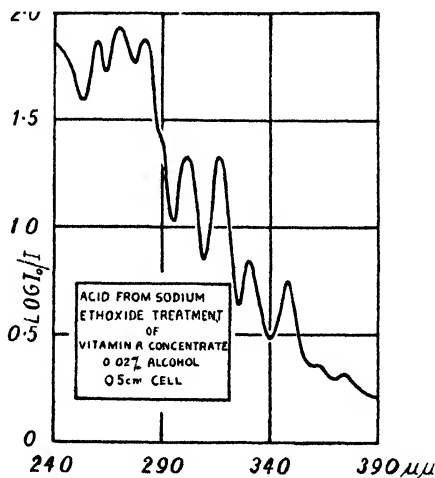


Fig. 5.

observed that this resembles the curve shown in Fig. 4 from which it appears that the characteristic selective absorption is due to acid material. The fact that this is shown in the original so-called neutral portion is due to the solubility of the sodium salts in ether, for by repeated washing of the ethereal solution, first with dilute alkali and then with water, a true neutral material was ultimately obtained in which the fine absorption bands had almost entirely disappeared; with antimony trichloride a violet-blue solution was obtained exhibiting a maximum of considerable intensity at 583μ .

Owing to the difficulty experienced in accomplishing complete decomposition of the vitamin in the above experiment, it was decided to work at a somewhat higher temperature and for this the alcoholic sodium ethoxide was replaced by sodium amyloxide. The material employed in this case was a cod-liver oil concentrate obtained from Messrs J. Nathan and Co., Ltd. The vitamin A potency was of the same order as that of the Lever extract, but the saponification equivalent in this case was 3900. The material was accordingly re-saponified with 5 % alcoholic potash; the product obtained had then a saponification equivalent of 8200, and was treated with sodium amyloxide in the following manner.

Sodium (12.5 g.) was dissolved in distilled amyl alcohol (125 g.), contained in a 1000 cc. round-bottomed flask fitted with a reflux condenser, and the solution was heated in an oil-bath at 180° in a current of nitrogen. The vitamin concentrate (25 g.) was added, and the oil-bath was kept at 180° for 2 hours,

after which the amyl alcohol was removed as far as possible under reduced pressure. The solid residue was then cooled in an atmosphere of nitrogen and heated with distilled water (250 cc.) until the sodium amyloxyde had been decomposed. The product was extracted with ether (1000 cc.), acetone being added to break the emulsions formed. The ethereal extract was repeatedly washed with water, and the washings were combined with the first aqueous extract. This was again extracted with ether until the soaps were entirely freed from neutral material. The alkaline solution was acidified with dilute sulphuric acid, saturated with salt and extracted with ether. The extract was dried over anhydrous sodium sulphate, giving on evaporation the acid portion (3 g.) again in amount considerably in excess of that calculated (0.9 g.) from the saponification equivalent taken as cod-liver oil glycerides. The spectrographic examination of the acid material gave the curve shown in Fig. 6,

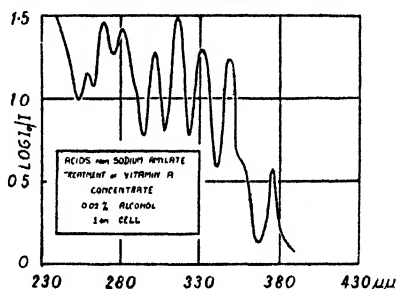


Fig. 6.

revealing the same series of bands as were obtained from the sodium ethoxide treatment. The intensity distribution differed in the two cases and comparison of the respective curves makes it highly probable that the acidic material is heterogeneous, consisting of at least three different selectively absorbing acids. The group of bands at 260, 271 and 282 μm appears to be due to one substance, the bands at 301 and 317 μm to another, those on the long wave side of 317 μm to at least one other acid, selective absorption beyond 260 μm remaining to be accounted for.

The production of these strongly absorbing acids next led us to examine spectrographically the total acids obtained by the ordinary saponification of cod-liver oil. We found here that the same type of absorption was shown by these acids [for curves see Gillam *et al.* 1931, pp. 34, 35], the ten characteristic bands being equally well-defined. At first sight it would thus appear plausible to suggest that the selective absorption finds its origin in the ordinary fatty acids; this view is however untenable for it fails to account for the absence of this complex absorption from cod-liver oil itself. Calculation of the intensity of the vitamin A band as compared with that of the acids rules out completely the suggestion that the acid bands are masked by the 328 μm band. Taking the intensity of absorption of a 1 % solution of cod-liver oil using a 1 cm. layer as unity, the intensity of absorption shown by the acids is about 50 times

as great. Masking being therefore eliminated an alternative hypothesis must be sought. Attention may be drawn to two significant points. In the first place it is highly improbable that the usual process of saponification would fail to hydrolyse any materials present in the fat as ordinary glycerides, and in the second place the amount of acidic material obtained from the concentrates by treatment with sodium ethoxide or amyloxide is largely in excess of that calculated from the saponification value.

The highly absorbing acids are therefore unlikely to be simple fatty acids and probably result from drastic and irreversible changes. The richest concentrates of vitamin A after treatment with sodium amyloxide did not exhibit the ten narrow bands, the decomposition products showing only one maximum at $276\mu\mu$ (Fig. 7). The acids cannot possibly be accounted for as arising solely

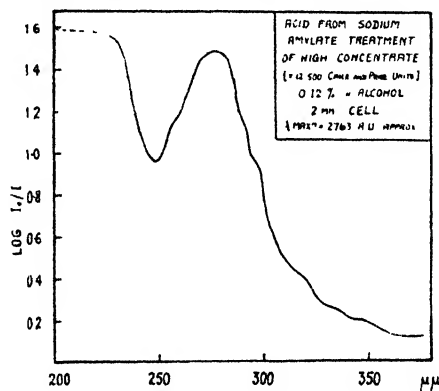


Fig. 7.

from the decomposition of vitamin A because the intensity of the new system of bands is no greater in the preparations from concentrates than in the total acids resulting from the ordinary saponification process, *i.e.* the accumulation of the vitamin in the concentrate is not accompanied by a corresponding increase in the amount of the highly absorbing acids. This experiment seems definitely to eliminate vitamin A itself as a sole contributory factor in the formation of the highly absorbing acids. The retention, in all but the highest concentrates, of material capable of yielding the acids possibly indicates that the bands are associated with either the later stages in the synthesis of vitamin A or the earlier stages of its utilisation in the organism. The band at $276\mu\mu$ may however well arise as a direct decomposition product of the vitamin, a suggestion supported by the appearance of such a band as a result of the photochemical destruction of vitamin A [Morton and Heilbron, 1928]. Control experiments on the action of sodium amyloxide on both ergosterol and dehydroergosterol have been carried out, and show that decomposition of these substances does not in any way account for the appearance of the absorbing acids.

We are therefore left to conclude that cod-liver oils contain appreciable

quantities of highly transparent substances which are decomposed during the process of saponification giving acids characterised by intense selective absorption. A much smaller quantity of not dissimilar, relatively diactinic material remains undecomposed and passes into the unsaponifiable extract. Drastic treatment with alkali is necessary before this residual, possibly more resistant, material is attacked, but when, as with sodium ethoxide or amyloxide, this occurs the acids produced are of the same type as before.

It is impossible not to be struck by a qualitative similarity between some of the groups of bands and the spectra of ergosterol and dehydroergosterol [Heilbron, Johnstone and Spring, 1929], and the fact that carotene shows a similar set of bands displaced into the visible [Dulière, Morton and Drummond, 1929]. The reappearance of well-resolved bands, again in groups of three, may well indicate that the acids are not unrelated to substances either of the poly-ene or sterol type.

SUMMARY.

1. Precise spectroscopic data regarding the vitamin A ultra-violet absorption band are recorded.
2. The vitamin A band at $328\mu\mu$ is found to be free from fine structure.
3. Nearly all cod-liver oils exhibit selective absorption in the region $260-295\mu\mu$.
4. Spectroscopic examination of the blue solutions obtained with antimony trichloride disclose, with cod-liver oils giving a clear ultra-violet band at $328\mu\mu$, a single sharp band at $604-608\mu\mu$.
5. With crude cod-liver oils of high potency additional selective absorption between $565-585\mu\mu$ is frequently observed in the blue solution.
6. The blue solutions given with concentrates have the main band at $620-624\mu\mu$ and in many cases show a less intense band at $582-593\mu\mu$.
7. Vitamin A is decomposed on treatment with sodium ethoxide. Concentrates so treated yield acids characterised by a series of well defined absorption bands with maxima near 394, 375, 350, 330, 316, 302, 282, 271 and $260\mu\mu$.
8. Similarly absorbing acids are produced by the ordinary saponification process, but evidence is adduced showing that these acids cannot be ordinary fatty acids and that they are not present as simple glycerides in the oil itself.

We desire to express our thanks to Messrs Lever Brothers, Ltd., and Messrs Joseph Nathan and Co., Ltd., for material used in this investigation, and also to the Department of Scientific and Industrial Research for a grant to one of us (A. T.).

REFERENCES.

- Capper (1930). *Biochem. J.* **24**, 453.
Collison, Hume, Smedley-MacLean and Smith (1929). *Biochem. J.* **23**, 634.
Drummond, Ahmad and Morton (1930). *J. Soc. Chem. Ind.* **49**, 291 T.
—— and Baker (1929). *Biochem. J.* **23**, 274.
—— and Morton (1929). *Biochem. J.* **23**, 785.
Dulière, Morton and Drummond (1929). *J. Soc. Chem. Ind.* **48**, 316 T.
Euler, Euler and Hellström (1928). *Biochem. Z.* **203**, 370.
Gillam, Heilbron, Hilditch and Morton (1931). *Biochem. J.* **25**, 30.
Heilbron, Johnstone and Spring (1929). *J. Chem. Soc.* 2248.
Moore (1929). *Biochem. J.* **23**, 803.
—— (1930). *Biochem. J.* **24**, 692.
Morton and Heilbron (1928). *Biochem. J.* **22**, 987.

V. SPECTROGRAPHIC DATA OF NATURAL FATS AND THEIR FATTY ACIDS IN RELATION TO VITAMIN A.

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(Received November 21st, 1930.)

THE observation that vitamin A concentrates yield, on treatment with sodium ethoxide, acids of which the absorption spectra exhibit characteristic fine structure [Morton, Heilbron and Thompson, 1931] has prompted us to commence a detailed investigation of the absorption spectra of natural fats and their related acids.

In the present paper certain broad aspects of the problem are considered.

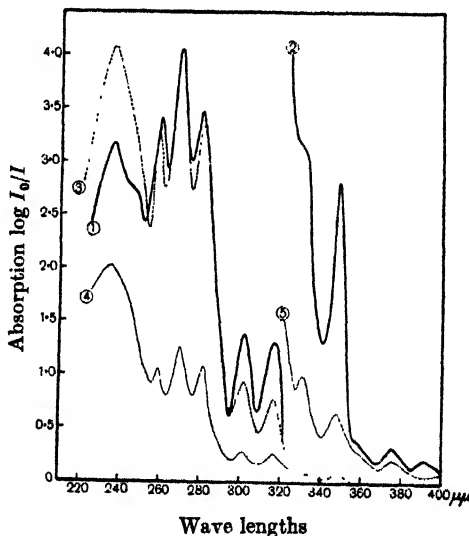


Fig. 1.

- (1) Total acids from cod-liver oil 0.08 % [2 mm.].
- (2) " " " 1.00 % [2 mm.].
- (3) Saturated and less unsaturated acids from cod-liver oil 0.4 % [2 mm.].
- (4) Synthetic glycerides of cod-liver oil acids 0.08 % [2 mm.].
- (5) " " " 1.0 % [2 mm.].

Attention has not been solely confined to vitamin A-containing oils but has been extended to embrace typical vegetable fats. In the first place the total

acids¹ from cod-liver oil have been examined. The absorption spectra of these are characterised by sharp bands with heads at about 392, 375, 350, 330, 316, 302, 281, 270, 259 and 235 $\mu\mu$ (Fig. 1, curves 1 and 2), and correspond closely with those of the selectively absorbing acids from the vitamin A concentrates [Morton *et al.* 1931, p. 24]. Fig. 2 (curve 2) gives the absorption spectrum of a

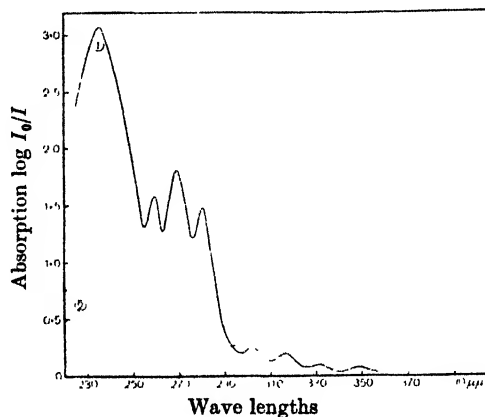


Fig. 2.

- (1) ——— Total acids from halibut-liver oil 0.2 % [2 mm].
 (2) Halibut-liver oil 0.2 % [2 mm].

halibut-liver oil rich in vitamin A (180 Carr and Price units) and here the smooth characteristic vitamin band at 328 $\mu\mu$ is clearly shown with, in addition, a second small band at about 225 $\mu\mu$. The acids obtained from this oil on saponification reveal on the other hand a wholly different type of absorption, bands with heads at identically the same positions as those of the cod-liver oil acids are now shown although a difference is observed in their relative intensities (Fig. 2, curve 1).

In Fig. 3 the absorption curve obtained for butter acids is given, butter being chosen to represent a non-liver animal fat containing vitamin A. It will be seen that five definite maxima appear, indicating the presence of small quantities of substances having the same bands as those shown by the cod- and halibut-liver oil acids. Turning now to butter-fat itself, this has been examined in chloroform solution and it is important to observe that in this case the spectrum shows distinct selective absorption at 322, 309, 284, 274 and 231 $\mu\mu$. There is little doubt that these bands correspond with those of the acids in alcohol, the shift towards the red being a solvent effect quite similar to that observed by Morton and Heilbron [1930] in the carotene bands of the non-saponifiable matter from butter. It is important to note however that, in striking contrast to butter itself, its non-saponifiable fraction does not give rise to narrow ultra-violet absorption bands.

¹ Unless otherwise specified, total acids include unsaponifiable matter originally present in the oil, the acids being prepared in the usual way by alkaline hydrolysis of the fat followed by liberation of the acids and extraction of the latter with ether. In the case of the cod-liver acids, the removal of the unsaponifiable matter brought about no alteration in the spectrum of the acids.

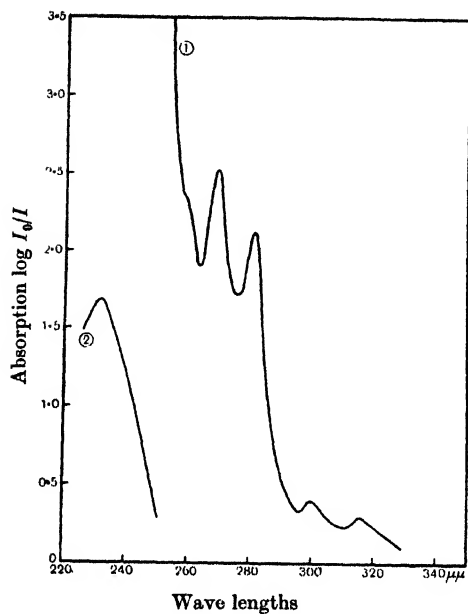


Fig. 3.

Total butter acids. (1) 1 % [1 cm.] alcohol. (2) 0.2 % [2 mm.].

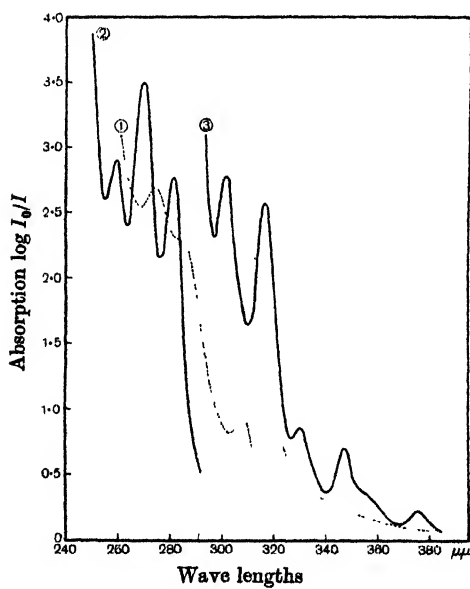


Fig. 4.

- (1) Whale oil 0.234 mm.
 (2) Acids from whale oil 0.25 % alcohol [5 mm.].
 (3) " " 1.0 % alcohol [1 cm.].

The case of whale oil was next considered, this being selected as a typical marine animal non-liver oil deficient in vitamin A. The absorption spectra of the mixed acids from this oil are, as shown in Fig. 4 (curves 2 and 3), qualitatively, and roughly quantitatively, comparable with the cod- or halibut-liver acids. The oil itself (curve 1) like butter, also has selective absorption similar to that of the acids, but different in intensity. This specimen of whale-oil contained nearly 1 % of free fatty acids; in order to determine whether these were responsible for the observed selective absorption they were removed and the neutral oil was again submitted to spectrographic examination. The same banded structure was again observed, but the intensity of absorption in the region 260-280 $\mu\mu$ appeared to be increased. It is thus clear that, as with butter, the selective absorption of the whale oil is not exclusively due to hydrolytic products of the fat.

In Fig. 5 (curves 1 and 2) the absorption spectra of the mixed fatty acids

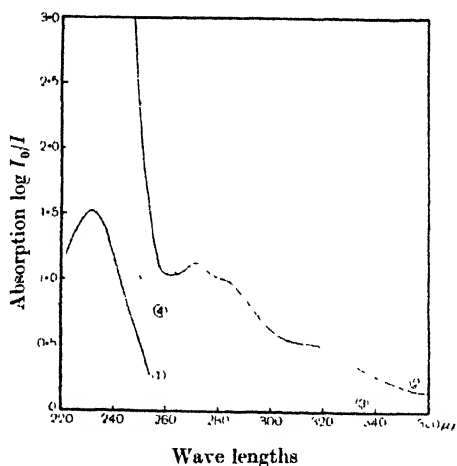


Fig. 5.

- (1) Thresher shark-liver acids 0.2 % [4 mm.].
- (2) " " 0.2 % [2 cm.].
- (3) Methyl esters of Thresher shark-liver acids 0.2 % [4 mm.].
- (4) " " 0.04 % [4 mm.].

of a Thresher shark-liver oil, from which vitamin A is absent, are reproduced. A comparison of the curves with those given in Figs. 1, 2 and 4 affords evidence of marked differences in definition and intensity. In place of the numerous sharp bands in the latter curves there are here only ill-defined maxima at 270, 280 and 315 $\mu\mu$; on the other hand, the prominent band at 230 $\mu\mu$ still persists¹.

Finally we have selected two examples from the vegetable oil group. The absorption spectra of olive oil and its mixed acids are shown in Fig. 6, while

¹ It should be noted that a band of high intensity in the extreme ultra-violet occurs with great regularity in a wide range of unsaturated organic compounds.

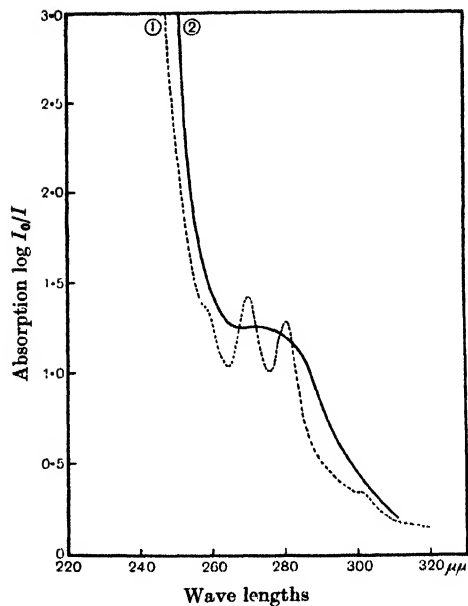


Fig. 6.

- (1) — Olive oil acids [1 cm.] 1 % alcohol.
 (2) - - - Olive oil [2 cm.] 1½ % alcohol.

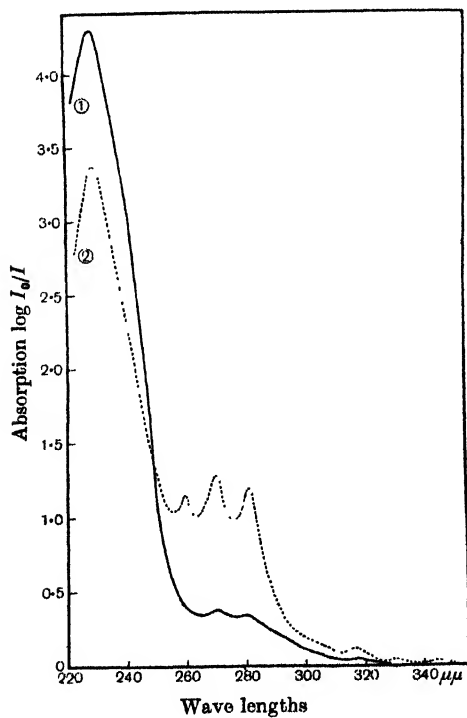


Fig. 7.

- (1) — Cottonseed acids 0.2 % [3 mm.].
 (2) - - - Cottonseed oil 1.0 % [2 mm.].

the curves for cottonseed oil and its acids are represented in Fig. 7. The curves differ markedly from those shown by the vitamin A-containing liver-oils and the corresponding acids of the latter; the vegetable oils exhibit a triplet group of bands with heads at 260 , 271 and $281\mu\mu$ and indications of other absorption in the region 300 - $350\mu\mu$, but the typical vitamin A band is absent. The curves for the corresponding mixed acids somewhat surprisingly show less fine structure between 260 - $280\mu\mu$ than those for the respective oils, and thus stand in striking contrast with those of the fish-liver oil acids with which this discussion was opened.

Although the present communication is mainly preliminary in nature and confines itself for the most part to a statement of experimental observations, it is nevertheless desirable to consider the evidence available as to the nature of the compound or compounds which give rise to the characteristic elaborate spectrum with 8-10 sharply defined absorption bands. It seemed possible at one time that the source of the banded absorption might lie in some of the highly unsaturated fatty acids common to marine animal oils but the experiments now to be described lend little support to this view.

The total mixed fatty acids from cod-liver oil (Fig. 1) were separated into

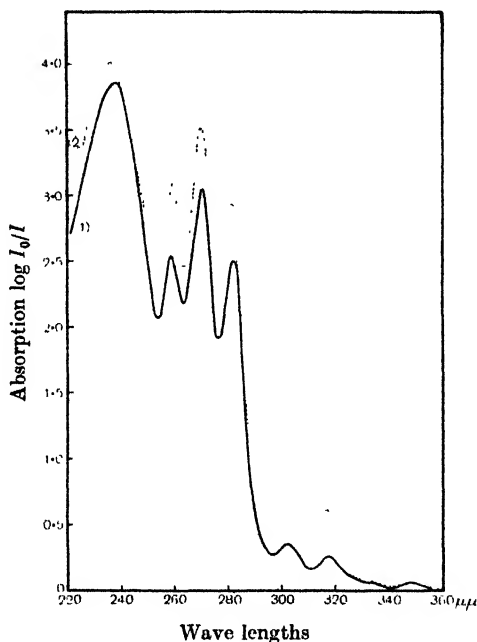


Fig. 8.

- (1) ——— Highly unsaturated acids from cod-liver oil 0.06 % [2 mm.].
 (2) - - - - - Methyl esters of above acids 0.05 % [2 mm.].

two groups by crystallisation of their lithium salts from acetone. The acids from the salts insoluble in acetone, which consist almost entirely of the

saturated and monoethylenic derivatives, have similar absorption (Fig. 1, curve 3) to that of the total mixed acids (Fig. 1, curves 1 and 2) but the intensity has been decreased by about 80 %. On the other hand with the acids from the soluble lithium salts which comprise the very highly unsaturated acids together with any unsaponifiable matter soluble in acetone, although the type of selective absorption is the same (Fig. 8, curve 1) the intensity is greater than with the total mixed acids. Fig. 8 (curve 2) shows the absorption curve of the corresponding methyl esters from the soluble lithium salts; this is in all respects similar to the curve of the free acids. These esters were submitted to fractionation under high vacuum as follows:

Fraction	Wt (g.)	B.P. (1 mm.)	Sap. equiv.	Iodine value
1	15.5	80-160°	283.8	138.2
2	41.6	160-177°	294.8	208.1
3	31.9	177-185°	307.0	266.4
4	46.2	185-190°	319.9	271.3
5	59.3	190-195°	333.7	285.4
6	25.4	195-200°	345.7	271.0
*Residue	84.0			

* Mainly polymerised highly unsaturated esters.

and the absorption spectrum of each of these primary fractions was observed. The following table gives the approximate relative intensity of the various bands calculated to 1 % solution in alcohol and 1 cm. thickness.

Wave length of maxima ...	235 $\mu\mu$	270 $\mu\mu$	281 $\mu\mu$	316 $\mu\mu$
Fraction 1	100	42	25	8
" 2	150	25	16	2
" 3	200	35	28	?
" 4	212	75	53	2
" 5	250	35	28	3
" 6	325	36	25	4

It will be observed that whereas the intensity of the band at 235 $\mu\mu$ (*cf.* footnote, p. 33) rises steadily throughout the series to a maximum, the bands at 270 and 281 $\mu\mu$ reach maximum intensity in fraction 4, but this intensity is already relatively much lessened as compared with that of the undistilled esters. Fraction 4 was re-distilled and the two main fractions having B.P. respectively 160-170° and 170-172°, and corresponding iodine values 279.8 and 301.5, were again examined. The absorption curves of these fractions are reproduced in Fig. 9 (curves 2 and 3). Comparison with the absorption of the esters prior to fractionation (curve 1) shows that a pronounced decrease in both absorption and selectivity has been brought about, presumably by decomposition. In view of these results it is improbable that the recorded fine structure can be attributed to esters of highly unsaturated acids ("clupanonic" type). Moreover, a similar even more unsaturated re-fractionated ester from the acids of Thresher shark-liver oil (sap. equiv. 327.3, iodine value 320.3) fails to show (Fig. 5, curves 3 and 4) the characteristic fine structure under discussion.

It might be argued that these results leave open the bare possibility that the observed selective absorption may be due to the presence of those highly

unsaturated esters of cod-liver oil which polymerise entirely [*cf.* Guha, Hilditch and Lovern, 1930], and which are left in the residue (84 g.) from the primary fractionation. If these were indeed the cause it is difficult to understand why the original oil is wholly devoid of the banded spectrum for if the acids were actually present in the oils themselves in the form of glyceryl esters there is no reason to suppose that, with glyceryl as with methyl esters, the same absorption should not be shown. We have in fact observed that the neutral triglycerides prepared from glycerol and the total mixed fatty acids of cod-liver oil by heating the alcohol with an excess of the free fatty acids under reduced pressure exhibit qualitatively the same absorption characteristics as the original fatty acids (Fig. 1, curves 4 and 5).

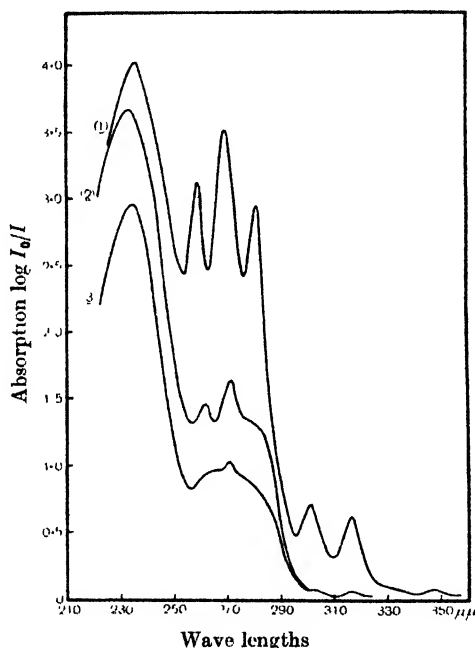


Fig. 9.

- (1) Methyl esters of highly unsaturated cod-liver oil acids 0.05 % [2 mm].
 (2) After refractionation; iodine value = 279.8
 (3) " " " = 301.5 } 0.05 % [2 mm].

DISCUSSION OF RESULTS AND SUMMARY.

In considering the data, a true perspective must be maintained. From the physiological angle vitamin A, vitamin D and ergosterol loom large, but from the point of view of quantitative analysis they are merely contaminants of minor constituents. This background cannot be dispensed with when the investigation of liver oils broadens out to embrace the indirect as well as the direct attack on the nature of the substances to which we attach special biological significance. Whilst the results now recorded form a logical sequence of the study of vitamin A, and it is natural to hope that a not very remote

connection may emerge, there seems to be no justification for allowing the discussion to be dominated by vitamin A.

It appears to us at the moment that the simplest explanation in consonance with all the facts so far observed is that under hydrolytic conditions a substance (or substances) accompanying vitamin A gives rise to acid decomposition products which display intense selective absorption. We suggest that even under the ordinary mild conditions of saponification, sufficient of this acidic decomposition product is formed to give rise to the observed spectra. That the acid decomposition products are in some way connected with the presence of vitamin A is indicated by the distinctly different absorption curves obtained with the acids from the non-vitamin A-containing olive or cottonseed oils.

It must again be emphasised that the absorbing acidic substances could not have been present originally in the form of simple esters for, were this the case, the liver oils should exhibit similar selective absorption. Indeed, once the acidic substances have been developed their characteristic spectra persist after their esterification with either methyl alcohol or glycerol. We are thus forced to the conclusion that their formation is the result of some form of molecular breakdown.

A most interesting corollary lies in the observations connected with the whale (blubber) oil examined and also with butter. With whale oil we are dealing with a vitamin-free fat which nevertheless originates in the liver, from which organ it is highly improbable that the vitamin is absent. If it be assumed that products of both hydrolytic and synthetic actions taking place in the liver are deposited in the tissue fat, an explanation is immediately forthcoming of the selective absorption of the oil as well as its derived acids. The same reasoning appertains in the case of butter, although here vitamin A itself is also present to a small extent in the milk fat.

The correctness of the foregoing speculations remains to be tested by the extended experimental work which we are undertaking on this subject. The main object of the present communication has been to record the evidence which we have collected showing that the fatty acids (or esters prepared therefrom) produced in the ordinary hydrolysis of vitamin A-containing liver oils, or of some other fatty oils from animals whose liver oils contain vitamin A, yield highly characteristic banded absorption spectra which are absent from the corresponding acids of vitamin A-free oils.

We desire to thank Dr G. Collin, Mr K. D. Guha and Dr A. Thompson for assistance in the preparation of a number of the compounds studied in the course of this work and the Department of Scientific and Industrial Research for assistance in various directions.

REFERENCES.

- Guha, Hilditch and Lovern (1930). *Biochem. J.* **24**, 266.
Morton and Heilbron (1930). *Biochem. J.* **24**, 870.
—— — and Thompson (1931). *Biochem. J.* **25**, 20.

VI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XIX. 6-HYDROXY-2-METHYLBENZOIC ACID, A PRODUCT OF THE METABOLISM OF GLUCOSE BY *PENICILLIUM GRISEO-FULVUM* DIERCKX.

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(Received November 28th, 1930.)

IN a series of publications which have recently appeared [Raistrick *et al.*, 1931] an account is given of an intensive study, extending over a period of seven years, of the products of the metabolism of glucose, in synthetic media containing no other source of carbon, by a large number of different species of the lower fungi. Several new types of metabolic products were described, many of which were shown to contain a benzene nucleus.

More recently it has been possible to extend this work, and, thanks to the courtesy of Professor Philip Biourge of the University of Louvain, several different species of *Penicillium* have been examined which were not available previously.

One of these species was *P. griseo-fulvum* Dierckx, which was originally described by Dierckx [1901], and, more recently, by Biourge [1923] in his monograph on the genus *Penicillium*. This species bears Biourge's catalogue number 34. In view of the interesting results we obtained with this species, we asked Professor Biourge to re-examine our culture and his report follows.

"Voici assez parfaitement déterminé le *Penicillium* que vous m'avez envoyé pendant les vacances. C'est bien le *Penicillium griseo-fulvum* Dierckx (Biourge No. 34). C'est une espèce peu commune. C'est la troisième fois que je la rencontre, y compris l'observation originale de Dierckx en 1898-1900, dans mon laboratoire. Elle est bien pure. Spores bleu céleste très pâle, et finalement gris-rosé. Revers jaune à jaune-rouge. Coremia tardifs, liquéfaction de la gélatine souvent précoce, odeur 0."

We desire to thank Professor Biourge for his friendly co-operation.

It was found that, when this species of *Penicillium* is grown on a synthetic medium containing glucose as the sole source of carbon, the metabolism solution, after some days, gives an intense purple colour with ferric chloride, very similar in shade to aqueous potassium permanganate solution. In addition the metabolism solution also gives a heavy, pale yellow precipitate with

bromine water. The isolation and identification of the substance responsible for these reactions is the object of the present communication.

Preparation of the metabolic product.

A quantity of a slightly modified Czapek-Dox medium was made up, having the following composition:

NaNO ₃	2.0 g.
KH ₂ PO ₄	1.0 g.
KCl	0.5 g.
MgSO ₄ ·7H ₂ O	0.5 g.
FeSO ₄ ·7H ₂ O	0.02 g.
Glucose (pure)	50.0 g.
Dist. water	to 1000 cc.

350 cc. quantities of this medium were placed in 67 1-litre conical flasks, which were plugged with cotton-wool, and sterilised by steaming for half an hour on each of three consecutive days. Each flask was heavily sown with an emulsion, in the above medium, of spores prepared from a 17-day culture of *P. griseo-fulvum* Dierckx, grown on Czapek-Dox agar at 25°. The flasks were thoroughly shaken and incubated at 25° for 35 days. At the end of this period the surface of the medium was completely covered with a thick mycelial felt, showing areas of pale green sporing patches, the intervening spaces consisting of sterile mycelium. The reverse of the mycelium was brick-red in colour. The contents of all the flasks were now filtered without previous sterilisation, the mycelium pressed and dried *in vacuo* at 50°. The combined filtrates, which were orange-yellow in colour, had the following characteristics.

- (1) Glucose (by polarimeter) 0.254 %.
- (2) Titratable acidity = 2.13 cc. *N* acid per 250 cc. medium.
- (3) Bromine absorption (by Koppeschaar's method) = 3.8 mg. per cc.
- (4) The metabolism solution gave a very intense purple colour with aqueous ferric chloride and a heavy pale yellow precipitate with bromine water.

The whole of the filtrate was acidified with 400 cc. of 2*N* H₂SO₄ and shaken with half its volume of ether, by which means the substance responsible for the ferric chloride reaction was completely extracted. The ethereal solution was washed with a little water, filtered and evaporated to dryness. The dry residue, which was brownish in colour and completely crystalline, weighed 26.9 g. corresponding to a yield of 2.42 % of the glucose consumed. It was crystallised from chloroform, from which solvent it separated in long white needles, which were practically pure but were recrystallised several times for analysis.

The dried mycelium was powdered and exhaustively extracted with ether. The ethereal extract on evaporation deposited a quantity of a white crystalline material which melted in a crude state at about 150°, gave a brownish purple

colour with ferric chloride, but is apparently not identical with the product extracted from the metabolism solution. The nature of this product will form the subject of a future communication.

Properties, analysis and derivatives of the metabolic product.

The product extracted from the metabolism solution and crystallised from chloroform has the following properties. It consists of beautiful white needles melting without decomposition at $170-171^{\circ}$ and may be readily sublimed unchanged in a high vacuum. It is not very soluble in cold water, but readily dissolves in hot water from which it separates on cooling in long white needles. It is very soluble in ether and alcohol, moderately soluble in hot, but not very soluble in cold, chloroform. Its solution in water or alcohol gives a very intense purple-violet colour with ferric chloride, very similar to that given by salicylic acid. Its aqueous solution gives an immediate precipitate with bromine water, and reacts acid to litmus. It gave the following results on analysis:

C, 62.91 and 63.02 %; H, 5.28 and 5.33 % (theoretical for $C_8H_8O_3$, C, 63.13; H, 5.30 %).

In an estimation of the molecular weight by the Rast camphor method, 0.217 mg. lowered the m.p. of 2.608 mg. camphor 20.2° , corresponding to a molecular weight of 160 (theoretical for $C_8H_8O_3$, 152).

Titration with $N/10$ sodium hydroxide to phenolphthalein gave an equivalent of 152.6 (theoretical for $C_8H_8O_3$, assuming this to be a monobasic acid, 152).

Methyl ether. 3 g. of the metabolic product were methylated by shaking with 9 cc. of dimethyl sulphate and an excess of 10 % sodium hydroxide. The mixture was maintained at room temperature for about 2 hours and was then boiled under a reflux condenser until perfectly clear. The solution was cooled, filtered, acidified and extracted with ether. The ethereal solution was evaporated to dryness and the dry residue, which weighed 3.1 g., was crystallised several times from boiling water. The crystallised material consisted of colourless, elongated hexagonal prisms (plates), which melted at 139° and gave no colour with ferric chloride. It gave the following results on analysis:

C, 65.39 %; H, 5.97 % (theoretical for $C_9H_{10}O_3$, C, 65.03 %; H, 6.07 %).

A Zeisel estimation gave 18.66 % OCH_3 (theoretical, 18.68 %).

Titration with $N/10$ sodium hydroxide to phenolphthalein gave an equivalent of 166.3 (theoretical for $C_9H_{10}O_3$, assuming this to be a monobasic acid, 166.1).

Acetyl derivative. 2 g. of the metabolic product, 3.3 cc. acetic anhydride and 6.6 cc. pyridine were mixed and incubated in a closed flask at 37° for 4 days. The mixture was cooled, diluted with 100 cc. of water, acidified to Congo red with $2N H_2SO_4$ and extracted with ether. The crystalline residue from the ether, weighing 2.5 g., was recrystallised several times from boiling

benzene, from which solvent it separated in colourless, elongated prisms, which melted without decomposition at 131° . This product which gave no colour with ferric chloride gave the following results on analysis:

C, 62.29 %, H, 5.23 % (theoretical for $C_{10}H_{10}O_4$, C, 61.83 %; H, 5.19 %).

0.2044 g. titrated with $N/10$ sodium hydroxide to phenolphthalein required 10.40 cc. giving an equivalent of 196.5 (theoretical for $C_{10}H_{10}O_4$, assuming this to be a monobasic acid, 194.1).

On account of the volatility of the metabolic product in steam, it was impossible to carry out a direct estimation of the acetyl groups, but this was done indirectly as follows. An excess of $N/10$ sodium hydroxide was added to the above neutralised solution, the mixture boiled for 3 hours under reflux, cooled and the excess of alkali titrated with $N/10$ hydrochloric acid. Acidity equivalent to 10.98 cc. $N/10$ was produced during the hydrolysis, from which it is evident that the derivative is a monoacetyl compound.

Constitution of the metabolic product.

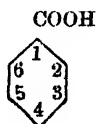
The above results indicate that the metabolic product has the empirical formula $C_8H_8O_3$, contains one hydroxyl group, which is probably phenolic in nature because of the purple colour produced with ferric chloride, and a carboxyl group. Since the material in aqueous solution also gives an insoluble bromine compound with bromine water, it is probably a benzene derivative.

Accepting this assumption for the moment, the metabolic product must be either (i) a hydroxyphenylacetic acid or (ii) a hydroxymethylbenzoic acid.

The possibility of the metabolic product being a phenylglycollic acid and having the hydroxyl group in the side chain is ruled out because of the reaction with ferric chloride.

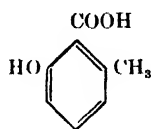
(i) The three possible hydroxyphenylacetic acids, 2-hydroxy-, 3-hydroxy- and 4-hydroxy- are known and melt respectively at $145-147^{\circ}$, 129° and 148° . The metabolic product melts at 171° and this, together with its general properties, proves that it is not a hydroxyphenylacetic acid.

(ii) There are ten possible hydroxymethylbenzoic acids, which have all been described. Writing the formulae for these according to the scheme



the metabolic product cannot be identical with any of the following, since they give no colour with ferric chloride; 5-OH 2- CH_3 ; 4-OH 2- CH_3 ; 3-OH 2- CH_3 ; 5-OH 3- CH_3 ; 4-OH 3- CH_3 ; 3-OH 4- CH_3 . This leaves the possibility that the metabolic product is one of the four methylsalicylic acids; 6-OH 2- CH_3 , 2-OH 3- CH_3 , 6-OH 3- CH_3 , 2-OH 4- CH_3 . All these acids give an intense violet colour with ferric chloride and melt respectively at $168-169^{\circ}$,

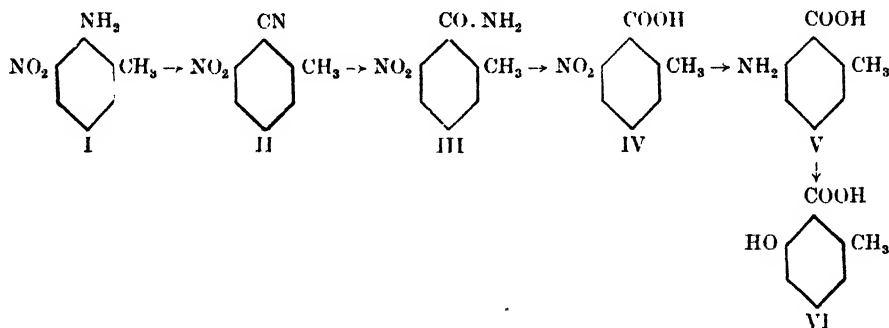
167°, 153° and 177°. (The highest recorded melting point is given in each case.) These melting points are not sufficiently far removed from the melting point of the metabolic product (171°) to be definitive. The melting points of the methyl ethers are, however, very much more widely separated and are respectively 139°, 85°, 70° and 104°. Since the melting point of the methyl ether of the metabolic product is 139°, it appears probable that the metabolic product is identical with 6-hydroxy-2-methylbenzoic acid of the following formula.



This conclusion was confirmed by synthesis.

Synthesis of the metabolic product.

We are indebted to Dr Alexander Robertson for synthetic specimens of 6-hydroxy-2-methylbenzoic acid, of its methyl ether and of its acetyl derivative. These were prepared as follows:



The nitrotoluic acid (IV) was prepared from the nitrotoluidine (I), *via* the nitronitrile (II), and nitroamide (III), according to the methods of Gabriel and Thieme [1919] and Kenner and Witham [1921].

The nitro-acid (IV) was reduced by means of ferrous sulphate and ammonia as used by Gabriel and Thieme. The amino-acid (V) was isolated by extraction with ether, and was converted into the hydroxy-acid (VI) without further purification. A solution of the amino-acid (6 g.) in 90 cc. *N*/2 HCl was cooled to 0°, treated with sodium nitrite (3 g.) and the mixture kept at 0° for 1 hour. 5 % sulphuric acid (40 cc.) was then added and the mixture was rapidly heated to 65° by plunging the vessel into a water-bath at 70–75°, and was maintained at this temperature for $\frac{1}{2}$ hour. The hydroxy-acid, contaminated with only traces of brown coloured material, quickly separated. Concentrated hydrochloric acid (40 cc.) was added to the cooled reaction mixture, and after 2 hours in the ice-chest the hydroxy-acid was collected, washed with water, and air-dried. Yield 3–3.5 g. It was purified by crystallisation from chloroform.

The synthetic hydroxy-acid gives the same colour reaction with ferric chloride as the metabolic product, has the same crystalline form, and the same melting point (170–171°). A mixture of the natural and synthetic products melts at the same temperature.

The methyl ether and the acetyl derivative of the synthetic hydroxy-acid were prepared by the methods previously described for the corresponding derivatives of the metabolic product.

The melting point of the methyl ether of the metabolic product, of the synthetic acid and of a mixture of the two was found to be 139° in all three cases. Similarly, the melting point of the acetyl derivative of the metabolic product, of the synthetic acid and of a mixture of the two was found to be 131° in all three cases, and hence identity of the metabolic product with the synthetic acid (6-hydroxy-2-methylbenzoic acid) is clearly established.

Identity of bromine compound.

It has been previously mentioned (p. 40) that the metabolism solution gives a heavy pale yellow precipitate with bromine water. In order to prepare a quantity of this material 5 litres of metabolism solution were treated with a slight excess of a saturated solution of bromine in water, allowed to stand overnight, and the precipitate filtered off. It was air-dried and extracted with ether. The ether solution on evaporation left a brownish residue consisting of rosettes of needles. A portion of this was recrystallised from light petroleum and then sublimed in a mercury vapour vacuum, giving a sublimate, at a bath temperature of 50–60°, of pale yellow needles, which melted at 84° and on admixture with a sublimed synthetic specimen of 2:4:6-tribromo-*m*-cresol had a mixed melting point of 83–84°.

It contained 69.6 % Br (theoretical for $C_7H_5OBr_3$, 69.5 %). Since the same substance is produced when bromine water is added to a dilute aqueous solution of the sodium salt of the metabolic product $C_8H_5O_3$, it is evident that this material is responsible for both the purple colour with ferric chloride and for the precipitate with bromine water given by the metabolism solution. This precipitate consists principally of 2:4:6-tribromo-*m*-cresol.

SUMMARY.

6-Hydroxy-2-methylbenzoic acid was isolated and identified as a product of the metabolism of glucose by *Penicillium griseo-fulvum* Dierckx.

REFERENCES.

- Biourge (1923). *La Cellule*, **33**, 164.
Dierckx (1901). *Ann. Soc. Sci. Bruz.* **25**, 88.
Gabriel and Thieme (1919). *Ber. deutsch. chem. Ges.* **52**, 1081.
Kenner and Witham (1921). *J. Chem. Soc.* **119**, 1452.
Raistrick and co-workers (1931). *Phil. Trans. Roy. Soc. Lond.* B **220**. (In the press.)

VII. ON THE BEHAVIOUR OF "INDIFFERENT" ELECTRODES WHEN USED FOR THE DETERMINATION OF OXIDATION-REDUCTION POTENTIALS IN THE PRESENCE OF HYDROGEN.

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(Received December 3rd, 1930.)

IN some observations on the reduction potential of cultures of anaerobic organisms recently reported [Lepper and Martin, 1930] we found that gold electrodes gave entirely different results from iridium electrodes if hydrogen was liberated during growth. The gold electrode remained poised by the feeble parahaematin-haemochromogen oxidation-reduction system present in the muscle fibres of the meat used in the medium. The iridium electrodes, on the other hand, were rapidly converted into hydrogen electrodes and reached the potential of the buffer solution surrounding the meat. In order to get a better understanding of the behaviour of these electrodes we have examined their potential in weakly poised and unpoised buffer solutions in the presence of nitrogen, hydrogen and oxygen.

Preparation of the electrodes.

The iridium electrodes were prepared by the method of Westhaver [1905], the glass being given two coats of iridium.

The gold electrodes were made by heavily gilding platinum plates electrolytically in a bath of gold cyanide. As soon as the platinum appeared to be well gilded the electrode was removed from the bath, dried and heated in the blowpipe. This process was repeated four or five times until the electrode was a deep gold colour after heating. By this means a substantial film of gold was alloyed on to the platinum before it was finally coated with electrolytically deposited gold. Electrodes made in this way were much less sensitive to hydrogen and oxygen than merely gilded platinum electrodes.

Experiments in weakly poised solutions.

The electrodes were placed in phosphate mixtures containing indigo-carmin of different molecular strengths. The air was expelled from the electrode vessel by nitrogen deprived of traces of oxygen by passing through a

solution of anthraquinol made by reducing an alkaline solution of anthraquinone with sodium hydrosulphite [Conant and Fieser, 1924]. To secure fine bubbles the gas was made to traverse a piece of kieselguhr filter candle as shown in Fig. 1. When the air had been displaced from the phosphate solution a few drops of weak sodium hydrosulphite were added from a burette to reduce the indigo-carmin in part and readings of the potential were taken until it had become steady. Hydrogen free from oxygen was now run through the apparatus until the change in potential of the electrodes which took place had again reached its limit.

When the concentration of indigo-carmin was $0.001 M$ the gold electrode remained perfectly stable on substituting hydrogen for nitrogen, but in a concentration of $0.0001 M$ the potential rose 8 mv.

With a concentration of $0.002 M$ indigo-carmin the iridium electrode showed a rise of 10 mv. and with a concentration of $0.0001 M$ the electrode had nearly reached the potential of the hydrogen electrode by the end of half an hour. The behaviour of the iridium electrode is shown in Fig. 2.

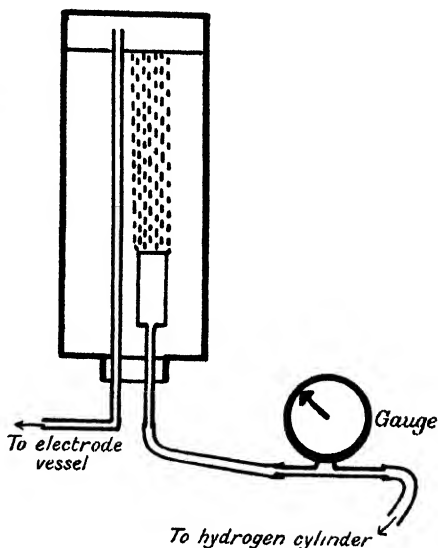


Fig. 1. Wash bottle for removal of oxygen from nitrogen and hydrogen.

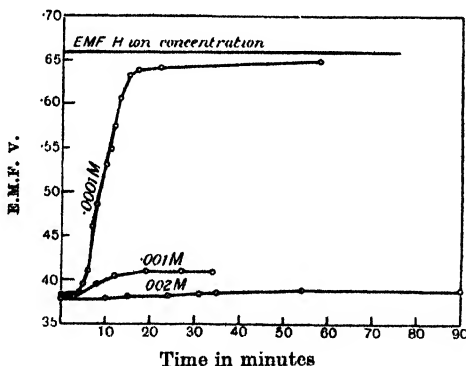


Fig. 2. The effect of hydrogen on an iridium electrode in different concentrations of indigo-carmin.

Experiments with unpoised buffer solutions.

A phosphate mixture was placed in the electrode vessel and the air was expelled by nitrogen. When sufficient time had been given to remove all the

oxygen from the solution hydrogen was substituted. At the end of $1\frac{1}{2}$ hours the negative drift of the gold electrode was 45 mv., whereas the iridium electrode had reached the potential corresponding to the hydrogen ion concentration of the solution, -0.7155 v., where it remained steady (Fig. 3).

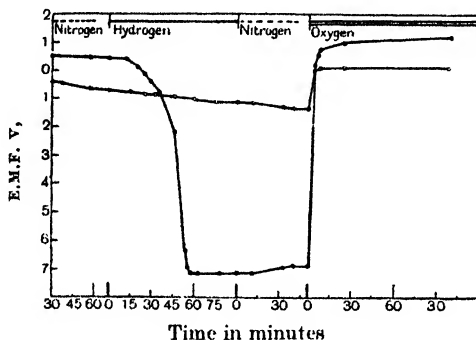


Fig. 3. The effect of hydrogen and oxygen on gold and iridium electrodes in phosphate buffer p_H 8.0.

..... Iridium.

o---o---o Gold.

The hydrogen was then displaced by nitrogen. After 45 minutes the gold showed a potential increase of 16 mv. and the iridium had fallen but 28 mv., indicating that the hydrogen absorbed by the iridium electrode is given off to an atmosphere of nitrogen only very slowly.

Lastly, when oxygen was run through the solution a rapid fall of potential occurred with both electrodes. At the end of 30 minutes the gold electrode again gave a steady potential. The iridium, on the other hand, after the first sudden fall to $+0.1$ v. continued to show a slow positive drift throughout the 5-hour period of observation.

The maximum potential of -0.125 v. observed at the gold electrode during the second period with nitrogen probably represents closely the potential of a really indifferent electrode at this p_H , as the oxygen present in the electrode at the time of immersion would, we presume, have been removed by the hydrogen.

It can be seen from Fig. 3 that gold appears to be much more sensitive to oxygen than it is to hydrogen.

DISCUSSION.

The explanation of the different behaviour of electrodes of gold and iridium during the growth of microbes when hydrogen is evolved appears to be that, the evolution of hydrogen being occasioned by an irreversible process and gold being relatively insensible to hydrogen, there is no reversible oxidation-reduction system present with a more negative range than the parahaematin-haemochromogen with which the gold electrode can come into electro-chemical equilibrium:

This interpretation is supported by the fact that when the more electro-negative dye safranine was added to the culture medium so as to make a concentration of about 0.01 %, in 1 hour after inoculation with *B. welchii* the E.M.F. afforded by the iridium electrode fell as before to that of the hydrogen electrode, but the potential of the gold electrode, instead of remaining poised by the parahaematin-haemochromogen dropped steeply until it was poised by the safranine around -0.33 v.

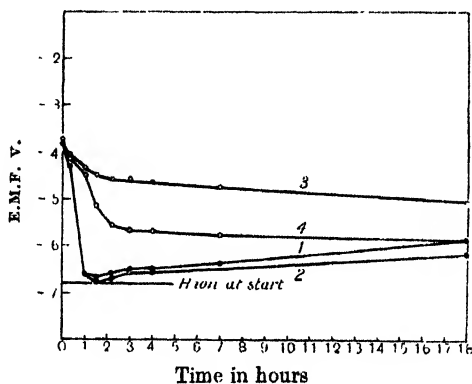


Fig. 4. The behaviour of gold and iridium electrodes when *B. welchii* grows in meat medium.

Curve 1. Iridium in meat. Curve 2. Iridium in meat containing safranine.

Curve 3. Gold in meat. Curve 4. Gold in meat containing safranine.

The iridium electrode behaves otherwise, because it becomes charged with hydrogen from the solution and functions as a hydrogen electrode. The reason for the hydrogen overvoltage noticed by Gillespie [1920] and also by Cannan, Cohen and Clark [1926] which also occurred in some of our experiments, is not obvious. The gas evolved contains less than 50 % hydrogen, but the conditions under which it is formed are conducive to supersaturation of the solution. Moreover, it is atomic hydrogen which is first separated, and this may find direct access to the electrode.

REFERENCES.

- Cannan, Cohen and Clark (1926). *Public Health Reports, Washington*, Supplement 55.
 Conant and Fieser (1924). *J. Biol. Chem.* **62**, 595.
 Gillespie (1920). *Soil Sci.* **9**, 199.
 Lepper and Martin (1930). *Brit. J. Exp. Path.* **11**, 100.
 Westhaver (1905). *Z. physikal. Chem.* **51**, 90.

VIII. THE FATTY ACIDS OF THE CAT'S KIDNEY. I.

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(Received December 3rd, 1930.)

INTRODUCTION.

WHEN the fatty acids which can be isolated from the cat's kidney are compared with those from the kidneys of other animals it is found that they present some striking differences. Whereas the mixed fatty acids from sheep or ox kidneys amount to about 2 % of the weight of the fresh organ and possess an iodine value of approximately 135, cat's kidneys yield about 4 % of fatty acids with an iodine value in the neighbourhood of 60. In spite of their lower iodine value, however, these mixed acids are semi-liquid whilst the more unsaturated acids from other animals are quite solid at room temperatures.

The earliest recorded values are those of Leathes and Meyer-Wedell [1909]. They found that the iodine values of fatty acids from the kidneys of normal cats were from 60 to 70, almost identical, that is, with the iodine value of 65 for the fatty acids from the adipose tissue fat. Mottram [1916] found that the average amount of fat in the kidneys of eight well-fed healthy cats was 5.11 % with an average iodine value for the fatty acids of 56, actually lower than the average iodine value of 61.4 for the adipose tissue fatty acids from the same cats. He also observed that the kidney tubules were heavily lined with stainable fat. Mottram suggested that the abnormally low iodine values were the result of an infiltration of fat into the kidneys, but for reasons which will be discussed later it seems improbable that this can be the correct explanation.

The kidney fat of the cat has been re-investigated with the results which are now to be described. •

EXPERIMENTAL.

Fatty acid estimations.

The low iodine values reported by Leathes and Meyer-Wedell [1909] and Mottram [1916] have been confirmed. It should be noted that Mottram's values refer only to the cortex of the kidney, but the following results show that the same is true for the whole kidney. The percentage of fatty acids in the kidneys was determined by the method described by Leathes and Raper [1925] and the iodine values were determined by the method of Wijs. Each

value given was obtained from the whole of one kidney; where two values are given they refer to left and right kidneys respectively. The iodine values were determined immediately after isolation of the fatty acids.

Table I.

Fatty acids (%)	Iodine value	Fatty acids (%)	Iodine value
3.72 3.69	82.6	6.37 6.83	71.7
4.28 4.61	84.1	5.70 5.48	71.0
5.40 6.54	69.3	3.65 3.78	87.7
4.14 4.37	80.5	3.21	97.8
6.77 6.73	69.6	5.68	76.0
5.58	63.5	2.75	113.0
5.63 5.92	69.5	2.16	98.0
6.30 6.18	88.7	4.15	79.0
5.29 4.76	79.9	3.58	87.4
4.07 4.06	86.5	6.83	53.2

Fatty acids: Max. value = 6.83 %.
 Min. value = 2.16 %.
 Mean of 32 = 4.9 %.

These values may be compared with those from typical beef kidney which had 2.09 % and 2.02 % fatty acids with corresponding iodine values of 133.8 and 131.0; and with the average for 34 human kidneys examined by Imrie [1914] of 1.9 % with iodine value 131.

EXAMINATION OF THE FATTY ACIDS.

A. *The isolation of the mixed fatty acids.*

The kidneys were collected from a large number of cats during a period of several months. Immediately after death the kidneys were removed from the body, carefully freed from all visible fat and connective tissue, weighed, cut into slices and placed in a small porcelain basin. The tissue was then saponified with a 50 % aqueous solution of caustic potash until completely liquid, methylated spirit was then added and heating continued until hydrolysis was complete. The resulting soap solution was poured into a stock-bottle. When about a kilogram of kidneys had been collected and saponified in this way, the accumulated soap solution was made strongly acid with 40 % sulphuric acid. After dilution with water to a suitable volume it was extracted three times with light petroleum. The extracts, which were deep red in colour, were collected in a flask and the solvent was removed on the water-bath in a slow current of carbon dioxide, the last traces being removed *in vacuo*. The crude fatty acids remaining in the flask were freed from unsaponifiable matter as completely as possible by repeatedly shaking out their solution in potassium hydroxide with light petroleum. The aqueous fluids were again acidified with sulphuric acid and extracted with light petroleum, the fatty acids being recovered by evaporation of the dried extract in a stream of carbon dioxide to prevent oxidation.

The mixed fatty acids formed a semi-liquid mass, yellow-brown in colour. If the flask containing them was left for some days in a tilted position, a liquid portion drained away from the solid acids. The whole readily liquefied

on very gentle warming. A peculiar musty smell was invariably associated with the mixed acids, though this may have been due to the presence of small amounts of impurities. The mixed acids usually had an iodine value about 56 and m.w. by titration about 274.

B. Separation of the mixed fatty acids.

The mixed fatty acids were separated into a liquid and a solid fraction by means of the lead soap-ether process.

The results of three distinct separations are summarised in Table II.

Table II. *Results of the separation of the fatty acids by the lead soap-ether process.*

Batch	Mixed acids		Unsaturated acids			Saturated acids	
	Iodine value	M.W.	Iodine value	M.W.	% of mixed	Iodine value	M.W.
A	56.9	264	62.5	263	61.5	4.4	—
B	56.4	274	67.3	274.2	60.6	5.8	276.4
C	56.4	274	68.7	282.4	63.9	14.0	275

The solid fraction (lead soaps insoluble in ether). These fatty acids were quite hard and solid, looking exactly like stearic acid. The m.w. of 274 combined with the very low iodine value suggests that they are mainly a mixture of palmitic and stearic acids in about equivalent proportions.

This is confirmed by the fact that an acid was obtained by repeated crystallisation of these acids from alcohol which had iodine value 0, m.w. 283.6 and m.p. 68.5°. 0.2280 g. acid neutralised 8.04 cc. *N*/10 NaOH; equivalent wt., 283.6. Stearic acid requires equivalent wt. 284, m.p. 69.2°, iodine value 0.

The liquid fraction (lead soaps soluble in ether). The acids composing this fraction were quite liquid even after standing for many months, although the iodine value was only 67.5. They were deep red in colour, but by boiling with charcoal in light petroleum solution they were finally obtained as a pale yellow oil. The peculiar musty odour was still apparent.

C. Further investigation of the liquid fraction.

The fluidity of this fraction combined with the very low iodine value (67.5) suggested the possible presence in the mixture of a liquid saturated acid. If this view were correct it should be theoretically possible to separate it from the liquid unsaturated acids by a preliminary hydrogenation of the mixture, by which these latter acids would be turned into solid saturated acids, followed by the usual lead soap-ether separation.

Hydrogenation of the liquid fraction. The hydrogenation was effected by the use of a platinum catalyst prepared by the method of Voorhees and Adams [1922]. This was found to be superior to a palladium hydrosol prepared by the method of Paal [1902, 1904]. Before the catalyst can be used for hydrogenations it must first be converted from the finely divided platinum

oxide into finely divided platinum black. A small quantity of the platinum oxide catalyst is shaken into a suspension in alcohol in a small round flask, the air is then swept out by a current of hydrogen from a Kipp's apparatus, and the suspension shaken in an atmosphere of hydrogen. In a short time a perfectly black, fine suspension is obtained. The fatty acids dissolved in a small volume of alcohol are then added to the flask containing the platinum-black catalyst and shaken for 2-3 hours in a hydrogen atmosphere under slightly more than atmospheric pressure. 26 g. of mixed liquid acids (iodine value 67.5) were in this way converted into 25 g. of hydrogenated acids which had the iodine value 7.8 and were quite solid.

Separation of the hydrogenated acids. The 25 g. of hydrogenated acids were next separated by the lead soap-ether process into (1) *a solid fraction*—a white hard solid, m.p. 69°, iodine value 0, m.w. 283.9, which therefore consists of pure stearic acid formed by hydrogenation of oleic and linoleic acids present in the liquid acids, and (2) *a liquid fraction*—7.5 g. of pale yellow mobile oil, having the iodine value 11.3, m.w. 268.

Esterification of the liquid acids. The 7.0 g. of liquid acids were dissolved in 50 cc. of methyl alcohol containing 3 cc. of sulphuric acid and heated on a water-bath for 2 hours. The liquid was then poured into a large excess of water and the esters were shaken out with ether. The ethereal extract was washed with water, then with potassium carbonate solution, again with water and finally dried over calcium chloride. Removal of the ether left 6.8 g. of a pale yellow, almost odourless, mobile oil.

The methyl esters were then distilled under a pressure of 15-20 mm. The first fraction distilled mainly at 105-115°, the temperature then rose fairly rapidly to 135° and the remainder of the esters distilled at 135-140°. The distillates were colourless faintly fatty-smelling oils. A dark brown, very viscous oil remained as a residue in the distilling flask, which after boiling for 2 hours with 20 % alcoholic potash remained unchanged. It was soluble in hot alcohol but insoluble in light petroleum or water.

Saponification of the ester fractions. The ester fractions were hydrolysed separately by boiling for 1-2 hours with 10 % alcoholic caustic potash. The resulting soap solutions were then acidified with H_2SO_4 and shaken out with light petroleum, and the extract was washed with water and dried. The fatty acids obtained on removing the solvent were from each fraction colourless, odourless, mobile oils.

Fraction (1). Wt. 1.2 g.

0.1938 g. neutralised 8.55 cc. $N/10$ NaOH. Equivalent wt. = 226.6.

0.1900 g. absorbed 0.2 mg. I_2 . Iodine value = 0.

$\text{C}_{14}\text{H}_{28}\text{O}_2$ requires equivalent wt. 228.

Fraction (2). Wt. 1.98.

0.1506 g. neutralised 6.14 cc. $N/10$ NaOH. Equivalent wt. = 245.3.

0.1322 g. absorbed 0.5 mg. I_2 . Iodine value = 0.

$\text{C}_{15}\text{H}_{30}\text{O}_2$ requires equivalent wt. 242.

0.16 g. of this acid dissolved in 10 cc. pure ethyl alcohol showed no optical activity.

The first fraction of these acids remained quite liquid when kept at a temperature of 0° for several days. The viscosity was rather higher than at room temperature but there was no sign of crystallisation. The higher boiling fraction on cooling to 0° deposited white, waxy crystals but the acids as a whole did not solidify. It seems that one acid crystallised out whilst another remained liquid. This fact, together with the knowledge that no C₁₅-acid is definitely known to be present in nature, suggests that the second fraction is probably a mixture of almost equal quantities of C₁₄- and C₁₆-acids. The C₁₆-acid would presumably be just solid at room temperatures.

D. Oxidation of the fatty acids from the cat's kidney.

The oxidation was carried out by two methods:

(1) *Hilditch's* [1926] *method*. Oxidation of the mixed acids by means of hydrogen peroxide (90/100 vols.) in glacial acetic acid at 70° as described by Hilditch gave rise to a dihydroxystearic acid, m.p. 92°. No sativic acid fraction was obtained.

(2) *Dilute alkaline potassium permanganate*. The mixed acids (iodine value 48) were also oxidised by dilute, ice-cold potassium permanganate in alkaline solution as was done by Hartley [1907] with acids from the liver.

Acids soluble in light petroleum formed 48 % of the original mixed acids. The iodine value was 27.3.

An acid insoluble in light petroleum, soluble in ether was obtained. This had m.p. 131° and was therefore dihydroxystearic acid.

An acid insoluble in light petroleum and ether, but soluble in boiling water, from which it crystallised on cooling, was also found. It had m.p. 164-166° corresponding with tetrahydroxystearic acid.

These two acids are derived from oleic and linoleic acids respectively. The presence of the latter acids in the fatty acids from the cat's kidney is therefore demonstrated.

E. Bromination of the acids from the cat's kidney.

1.38 g. of the liquid acid fraction (iodine value 67.5) were dissolved in 30 cc. of ether, and a 1 % solution of bromine in ether was added drop by drop to the ice-cold solution until excess of bromine was present. The solution was allowed to stand overnight in ice. A small white deposit was obtained which was filtered off through a weighed Jena glass crucible.

The ether was removed from the filtrate and the residue after weighing was treated with light petroleum (b.p. 60-80°). A dark brown precipitate was obtained; this was likewise filtered off and weighed.

1.38 g. of mixed liquid acids gave 1.85 g. bromination products, 0.02 g. insoluble in ether, 0.11 g. insoluble in light petroleum (b.p. 60-80°) and 1.72 g. of material (by difference) soluble in light petroleum.

Rollett [1909] has shown that about half of the linoleic tetrabromides produced from ordinary linoleic acid are soluble in light petroleum. Allowing for this, about 0.25 g. of the bromides out of the 1.85 g. are tetra- or poly-bromides. When it is considered that about 60 % of the weight of a tetrabromide is bromine, it is obvious that the amount of acids more unsaturated than oleic acid in the 1.38 g. of mixed acids must be very small. It is safe to conclude therefore that nearly all the iodine absorption of the liquid acid fraction is due to the presence therein of oleic acid.

The lipid fractions of cat's kidney.

A number of cat's kidneys were first exhaustively extracted with acetone and the residue was then extracted with ether. The two lipid fractions obtained correspond approximately with the glycerides and the phospholipins respectively. For comparative purposes a similar treatment was given to a quantity of beef kidney and the results of the investigations on these fractions are summarised in Tables III-V.

Table III. *Appearance of extracts.*

Fractions	Cat	Beef
(1) Acetone extract	Deep orange viscous oil	Deep orange wax
(2) Ether extract	Pale orange semi-solid	Pale orange waxy extract
1a. Fatty acids from saponification of Fraction (1)	Liquid	Solid
2a. Same from Fraction (2)	Waxy solid	Solid

Table IV. *Distribution of lipins.*

Fraction	Cat kidney (1) %	Cat kidney (2) %	Beef kidney %
Acetone extract	3.01	1.85	0.87
Ether extract	1.99	2.00	1.26

Table V. *Iodine values.*

Fraction	Cat kidney (1) %	Cat kidney (2) %	Beef kidney %
Acetone extract:			
Before saponification	55.0	69.3	117.9
Fatty acids from saponification	56.7	63.9	130.2
Ether extract:			
Before saponification	67.2	59.8	84.0
Fatty acids from saponification	74.9	55.5	95.2

DISCUSSION.

Mottram [1916] considered that the abnormally low iodine value of the cat kidney fat was due to a process of fatty infiltration of the kidney. This view was based on the fact that the cat kidneys contained, on the average, about 5 % of fat compared with the more usual value of 2-3 % for other animals, and that sections of the kidney stained for fat showed an abnormally large number of fat droplets lining the tubules. Assuming that 3 % of the

fats had the customary iodine value for organ fat of 135, which is true for most animals, then the additional 2 % represents infiltrated fat. If the infiltrating fat is adipose tissue fat, and it is to be presumed that Mottram intended this, then the iodine value of the mixed acids would be theoretically 107. This is greatly in excess of the observed iodine value of approximately 60 for the fatty acids from the cat's kidney. The infiltration of adipose tissue fat of iodine value 65 could obviously never account for an iodine value of the mixed acids lower than this value, yet in practice values as low as 50 are observed. It is found experimentally that the iodine value of adipose tissue fatty acids from cats is always about 65. For Mottram's view to be correct, therefore, it must mean that the iodine value of the fatty acids of the "élément constant" for the cat's kidney must be considerably below the accepted normal value of 135. In any case an infiltration hypothesis would not account for the remarkable fluidity of the acids from the cat's kidney. Adipose tissue fatty acids at room temperatures are quite solid, whereas the renal fatty acids of the same or even lower iodine value, are semi-liquid. There must be some fundamental difference in the nature of the fat in the kidney of the cat compared with that of other animals, for it is impossible by making use of values which are true for the latter to account at once for the lower iodine value and the more liquid nature of the fatty acids obtained from the cat's kidney. The presence of some fatty acids which possess a very low or zero iodine value and a high fluidity, for example liquid saturated acids, would reconcile these observations. The experimental procedure was, in consequence, directed towards the isolation of such acids if these were present. The actual isolation of completely saturated fatty acids which are liquid at room temperature confirms the truth of the above suggestion. The very limited amount of these acids at present available makes it impossible to define their constitutions. It is hoped however to proceed with the investigation of this problem when sufficient quantity of the material has been collected; in this preliminary investigation the loss of material has been very heavy, but for the future preparations it is believed that the acids will be isolated in greater yield by a much simpler process.

So far as is known the only naturally occurring liquid saturated fatty acids with more than fourteen carbon atoms are the tuberculostearic and phthioic acids found by Anderson [1929] and Anderson and Chargaff [1930] among the fatty acids from tubercle bacilli. These acids are isomeric with stearic and cerotic acids respectively. Branching of the chain may be a possible explanation of the liquid nature of these acids, since for example α -amyl-*n*-nonoic acid is still liquid at -10° and β -butyl-*n*-decoic acid has M.P. 4° .

The experimental evidence suggests that these liquid saturated acids are present in both the simple glyceride and phospholipin fractions of the fats in the cat's kidney. Both these fractions are noticeably more liquid than the corresponding fractions from beef kidney although the iodine values of the latter are much higher. The differences in iodine value are more marked in

the simple glyceride fraction, the values from the beef kidney being almost double those from the cat's kidney. On saponification, beef kidney gives rise to solid acids from both the simple glyceride and phospholipin fractions. The glyceride fraction from cat's kidneys gives rise on saponification to acids which are mainly liquid, whilst the phospholipins yield a waxy product. The inference is that it is in the simple glycerides that the larger proportion of liquid saturated acids is present. The solid saturated acids are apparently present to a greater extent in the phospholipins.

The occurrence of this type of liquid saturated fatty acids in the cat's kidney raises questions of no little physiological interest, but a discussion of these may more appropriately be reserved for a future communication.

SUMMARY.

The percentage amounts and iodine values of the fatty acids from the kidneys of 20 cats have been determined. The mixed fatty acids obtained by saponification of the kidney fats have been examined. They are shown to consist of palmitic, stearic, oleic and linoleic acids, together with at least two new liquid saturated acids. The constitutions of the latter have not yet been ascertained. A comparison has also been made between the simple glyceride and phospholipin fractions obtained by the same processes from cat and beef kidneys.

REFERENCES.

- Anderson (1929). *J. Biol. Chem.* **83**, 169.
— and Chargaff (1930). *J. Biol. Chem.* **85**, 77.
Hartley (1907). *J. Physiol.* **36**, 17.
Hilditch (1926). *J. Chem. Soc.* 1828.
Imrie (1914). *J. Path. Bact.* **19**, 245.
Leathes and Raper (1925). The fats, p. 58. (Longmans, Green and Co., London.)
— and Meyer-Wedell (1909). *J. Physiol.* **38**; *Proc.* xxxviii.
Mottram (1916). *J. Physiol.* **50**, 388.
Paal (1902). *Ber. deutsch. chem. Ges.* **35**, 2205.
— (1904). *Ber. deutsch. chem. Ges.* **37**, 133.
Rollett (1909). *Z. physiol. Chem.* **62**, 410.
Voorhees and Adams (1922). *J. Amer. Chem. Soc.* **44**, 1397

IX. THE PERMEABILITY OF HUMAN EPIDERMIS TO ULTRA-VIOLET IRRADIATION.

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THE work of Rosenheim and Webster [1927] and Windaus and Hess [1927] has shown that the irradiation of ergosterol with ultra-violet rays produces vitamin D. Since Hess, Weinstock and Helman [1925] found irradiated lanolin to be antirachitic, it may be concluded that ergosterol or a similar sterol is associated with cholesterol in the sebaceous material of the skin.

It has been shown by Hume, Lucas and Smith [1927] that ordinary impure cholesterol after irradiation, if gently rubbed into the intact shaven skin, can protect rabbits on a rachitogenic diet from rickets. The cholesterol was dissolved in ether and the solution poured on to a glass plate, and irradiated under a mercury vapour quartz lamp. The irradiated cholesterol was mixed with cottonseed oil to facilitate inunction, and rubbed into shaved areas on the backs of rabbits, these areas being covered with wash-leather jackets to prevent ingestion by the mouth.

Absorption through the skin of vitamin D, produced in skin fat, may not however be the only, or the usual, way in which the antirachitic effect of ultra-violet light is communicated to the animal body. Rosenheim and Webster have shown that only 0.0001 mg. of irradiated ergosterol daily is required to protect a rat from rickets or even to cure it. Moreover the amount of ultra-violet irradiation which reaches the surface of the rat is very small, since, with the exception of the ears, feet and horny tail, the rest of the rat is covered with fur; yet it is enough to protect the rat from rickets. It may be that enough ultra-violet rays of sufficiently short wave-length to activate the ergosterol may penetrate the epidermis and reach the ergosterol contained in the capillaries lying just below. The epidermis on the parts of the human body other than the palms of the hands and soles of the feet, is approximately 50μ thick at the peaks of the dermis where the network of capillaries is thickest.

PREVIOUS OBSERVATIONS.

Our knowledge of the permeability of skin and epidermis to ultra-violet light of different wave-lengths largely depends on the observations of Hasselbalch [1911] made 20 years ago, using light of wave-lengths ranging between 437μ and 289μ . His conclusions are those commonly referred to in the

literature. Hasselbalch's method was to use skin from a corpse, placing it between two discs of optically flat clear quartz so fitted into a short brass cylinder as to form an air-tight chamber in which the skin was pressed flat between the discs. He used a Kromayer quartz mercury vapour arc lamp and a quartz spectrograph. The skin was placed 5 cm. in front of the slit of the spectrograph which was widely open. To receive the photographic image he used Solio white matt printing-out paper. The time necessary to produce a suitable image through the skin was recorded. The dark-slide was then lowered by the rack and pinion and the illumination repeated through the quartz discs only, the image being received immediately above the preceding one and the exposure being continued until the two images of a selected mercury vapour band were of equal density. Owing to the use of Solio paper it was possible, without spoiling the image produced, to interrupt the exposure from time to time, to examine both photographs and stop the exposure at the desired point. When two images of equal density of one of the mercury-vapour bands of longer wave-length had been obtained and the respective times recorded, the same piece of paper could be replaced and the exposures continued for bands of shorter wave-length.

In the calculations which were the outcome of his experiments, Hasselbalch made use of absorption coefficients, calculating these according to the formula

$$\frac{J}{J_0} = 10^{-\epsilon' d} \quad \text{or} \quad \frac{-\log \frac{J}{J_0}}{d} = \epsilon'$$

where ϵ' is the coefficient of absorption, J is intensity of the transmitted light, J_0 that of the incident light, and d is the thickness of the skin in millimetres. The modern practice is to use centimetres, but Hasselbalch used millimetres and to render comparison easier his practice has been followed in presenting my results. The values for the transmitted and incident light are inversely proportional to the times of exposure required to produce an image of the same density. Thus

$$\frac{J}{J_0} = \frac{t'}{t}$$

where t and t' are the times required to produce images of the same density from transmitted and incident light respectively.

The absorption coefficients for skin, calculated by Hasselbalch, varied from 2.3 for a wave-length of $437\mu\mu$ to 5.2 for a wave-length of $313\mu\mu$ which was the shortest he was able to examine. For epidermis his figures were:

Wave-length	404	360	334	313	302	294	289 $\mu\mu$
ϵ'	2.3	4.2	6.6	8.5	12.4	17.5	39

Anderson and Macht [1928, 1, 2] made two series of experiments on the penetration of ultra-violet rays through living skin. In their first series they irradiated the skin of a living anaesthetised rabbit, reflected from the body, with monochromatic light from a mercury vapour arc lamp. They recorded

the energy transmitted through the skin by means of a thermopile and galvanometer. The result showed that between the wave-lengths $360\mu\mu$ and $280\mu\mu$ the percentage transmission increased from 11.4 % to 56.3 % and that there was then a drop to 23 % at $265\mu\mu$ with a further rise to 42.8 % at $253\mu\mu$.

This paper was criticised by Hill [1928] and by Bachem [1928]. Hill's criticism is chiefly based on the results of his own physiological experiments. He showed the great protection against ultra-violet irradiation afforded by a layer of omentum which is far thinner than skin, and also the protection to tubercle bacilli afforded by a thin layer of serum. Bachem [1928] drew attention to the possible influence of stray light rays of other wave-lengths due to the imperfect production of monochromatic light, and to the error due to the fluorescence of the skin. These fluorescent waves affected the thermopile, and the energy thereof was registered as energy from ultra-violet waves coming through the monochromator.

Anderson and Macht in their second paper [1928, 2] used a special photometer to control the thermopile method and found that the amount of stray light was negligible. They admitted the error due to the fluorescence of the skin and corrected it. Nevertheless their findings show a rise of transmission from 7.5 % to 9.8 % between the wave-lengths $302\mu\mu$ and $280\mu\mu$. This is contrary to the findings of workers on the ultra-violet absorption of other protein substances.

It is not out of place at this juncture to point out that the error due to the inclusion of fluorescent rays can almost certainly be excluded when the transmitted rays are analysed by the spectroscopic method instead of a thermopile. In this method any fluorescent light from the skin is recorded on the photographic plate as visible light and not as ultra-violet. A source of error would only be possible if a proportion of the incident light were converted into light of a shorter wave-length by fluorescence. This form of conversion has not yet been established.

METHODS USED IN THE PRESENT WORK.

Two years ago I repeated Hasselbalch's experiments with human skin, but using parallel light. The results obtained, expressed as absorption coefficients per millimetre, indicated that the skin used was 100 to 10,000 (according to the wave-length) times more opaque than that used by him. In searching for a solution of this discrepancy the conclusion was reached that it was due to the loss of intensity of the issuing light caused by scattering, owing to the different conditions of our experiments.

Skin is not optically homogeneous. Incident light, in addition to being diffusely reflected at the surface, is irregularly refracted as it passes through the layers of cells which compose it. When therefore skin is interposed between the light-source and the slit of the spectrograph, many of the rays are reflected and the remainder so much bent that they miss the slit altogether, while others pass through at such an angle that they impinge on the wall of the collimator

tube and not on the prism. The result is that only a fraction of the incident light which is unabsorbed by the skin is recorded and measured against the incident light. If the incident rays are parallel the effect of scattering is maximal, and this is the reason why my early figures are higher than those of Hasselbalch, who apparently did not use parallel light.

When light impinges on the skin of a living animal scattering is not of such great importance, because the transmitted light does not subsequently encounter a narrow slit. Although much diffuse reflection occurs at the surface, rays of light striking the skin surface at one point ultimately reach the blood vessels, though possibly at positions widely separated from the point of incidence.

Another explanation of the exaggerated value for the absorption of skin obtained in experiments of this type is that the light traverses a devious path, the length of which is not necessarily represented by the thickness of the skin, which is the measure used for the calculation of absorption coefficients. It is not therefore possible to determine the absorption coefficient, which depends for its estimation on the thickness of the layer actually traversed. For this reason the expression of the absorption of light by the skin by means of absorption coefficients has been abandoned in these experiments, and the degree of absorption for light of different wave-lengths is expressed in terms of relation between the logarithms of incident and transmitted light respectively, $(\log \frac{J}{J_0})$, as measured by the respective times of exposure needed to obtain images of equal density after passage through the particular thickness of skin or epidermis used. As was stated previously the times of exposure are inversely proportional to the respective intensities of the light,

$$(\log \frac{J}{J_0} = \log \frac{t'}{t}).$$

In order to minimise the effect of scattering of light, it was decided to insert scattering surfaces in front of the light-source in the form of ground quartz discs, through which the light would pass in tests both with, and without, the skin. The skin was placed between two clear quartz discs as before (Fig. 1, 1 and 1'). One of these was recessed into an outer casing (2), with a fine screw thread on the outer side, while the other disc was similarly recessed into a thick ring of metal (3), which slid into (2), and fitted sufficiently well to make the cavity air-tight. The whole of this was then screwed into an outer casing (4), with a thread on the inner side. The scattering of the incident light was produced by two quartz discs each ground on both surfaces (5 and 5') fixed with plasticene on to the inner and outer surfaces of the front surface of (4). The whole then slid into the hood of the spectroscope from which the front cap had been unscrewed.

The light-source was a Hewittic quartz mercury vapour lamp working at 2.25 amperes. It was enclosed in a metal case and the light emerged through an aperture $1\frac{1}{2}$ inches in diameter.

The introduction of the ground quartz discs reduced the intensity of the light incident on the slit to such a degree that considerably longer exposures were needed. For this purpose a photographic shutter was made of a piece of flat metal blackened on both sides attached to the long arm of a lever, the short arm of which was operated by the armature of an electromagnet. This was placed in front of the cell holding the epidermis. When an electric current passed through the magnet the armature was pulled down, and the shutter on the long arm raised, thus making the exposure. The duration of the exposure depended on the length of time the current flowed. The timing apparatus consisted of a wooden pulley geared to a gramophone motor in a ratio

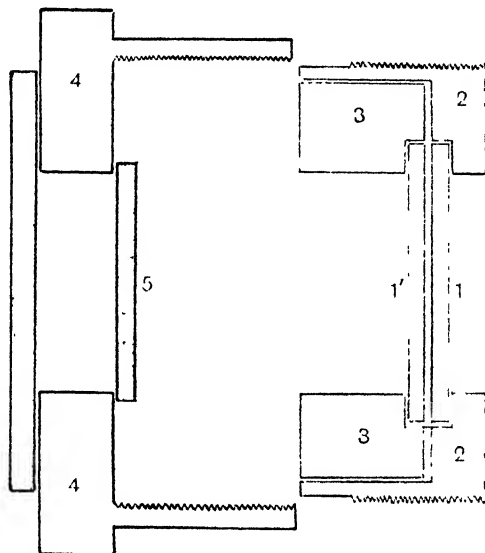


Fig. 1. Air-tight cell arranged to hold epidermis.

which produced one revolution in 5 seconds. A series of wooden discs into each of which was recessed a brass sector was attached to the face of the pulley. Two strips of springy brass were fixed so as to wipe against the face of the disc on the pulley and complete the circuit during the time they passed over the brass sector. The ends of the strips were in the line of the radius of the pulley face, and therefore reached the edge of the brass sector simultaneously. The shutter by its own weight fell back in front of the cell. Sectors were made to give exposures of from 1/10 sec. to 3 sec., varying by 1/10 sec. Instead of Solio paper, used by Hasselbalch, Ilford "Process" plates (H & D = 50) were employed.

The constancy of the source of illumination was examined by taking a series of spectrographs with the same period of exposure, one below the other, on the same plate. It was found that after the lamp had been lit for 30 minutes the light was so steady that there was no discernible difference of intensity between any of the spectrographs.

With a piece of human skin in position a series of exposures was made on a plate, varying between 1 second for the long wave-lengths and 30 minutes for the short ones. These provided the measure of the light transmitted through the skin or epidermis, which was then removed. With the cell otherwise the same, shorter exposures were made on further plates by means of the electromagnet shutter. These gave the measure of the incident light.

The two series of plates were developed for an equal time with developer at the same temperature. Then for each wave-length the intensity of a line in the series obtained from the transmitted light was matched with that of the same line in the series obtained from the incident light. The times of exposure of the two were then noted. If no two images showed exact equality of intensity, the average was taken between the two exposures of incident light, which gave images of the selected line of intensity respectively greater and less than the intensity of that from the transmitted light.

A trial of this apparatus showed that the diminution of the intensity of the incident light by scattering at the ground quartz surface was so great that no image of any line of shorter wave-length than $313\mu\mu$ could be obtained through skin of 0.2 mm. thickness except by exposures of 24 hours and longer.

Skin was therefore abandoned in favour of epidermis, a change the more justifiable, because it is only the thin covering of epidermis (of thickness about 0.05 mm.) which in the living body is interposed between the dermal capillaries and the source of illumination. Epidermis was obtained from the front of the fore-arm by means of a blister produced with chloroform vapour. Transverse sections, appropriately stained, showed that the stratum granulosum was included in the separated portion. These pieces of epidermis were stored in a refrigerator in tap-water, and when placed in the cell were moistened with tap-water.

EXPERIMENTS WITH HUMAN EPIDERMIS.

1. *Diminution of error due to scattering of light by interposing ground quartz discs.*

An experiment was made with a specimen of human epidermis 0.08 mm. thick and values for $\log \frac{J_0}{J}$ were obtained for wave-lengths corresponding to the chief lines in the violet and ultra-violet parts of the emission spectrum of the mercury vapour arc, *i.e.* from wave-length $437\mu\mu$ to $256\mu\mu$. In the first experiment (*a*) the incident light was previously scattered by the insertion of ground quartz discs as described above. A second experiment (*b*) was then performed with the light made approximately parallel. In order that images of submaximal intensity should be obtained for long wave-lengths of the incident light, the lamp was placed at a distance of 11 feet from the spectrograph. The resulting figures are shown in Table I, cols. *a* and *b*, and are reproduced graphically in Fig. 2, *a* and *b*, where the ordinates are values of $\log \frac{J_0}{J}$ and the abscissae are wave-lengths expressed in millimicrons.

Table I. *Absorption of violet and ultra-violet radiations by human epidermis 0.08 mm. thick.*

Wave-length in $\mu\mu$	Values of $\log J_0/J$	
	(a) Using light scattered by ground quartz discs	(b) Using parallel light
437	0.55	1.68
404	0.62	1.82
360	0.76	2.14
334	1.02	2.52
313	1.14	2.93
302	1.40	3.28
294	1.66	3.55
289	2.44	—
264	2.80	—
256	2.66	—

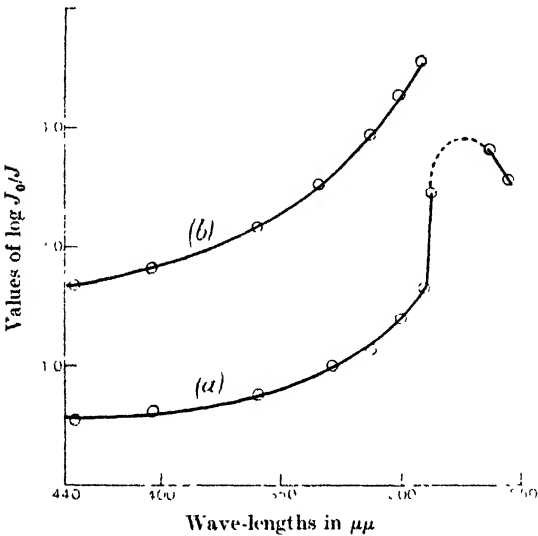


Fig. 2. Effect of using incident light scattered by ground quartz surfaces. Absorption curves of human epidermis (0.08 mm. thick).
a. Incident light scattered. b. Incident light parallel.

The apparent increase in the transmission through the epidermis when the incident light was previously scattered was unexpectedly great, being from 13 (for wave-length $437\mu\mu$) to 78 (for wave-length $294\mu\mu$) times greater than in Exp. b, where parallel light was used. As the same piece of epidermis was used in both cases, this difference is only an apparent one, due not to the epidermis, but to the method of illumination, the only thing changed in the two experiments. For wave-lengths less than $294\mu\mu$ the scattering by the epidermis becomes so great, when parallel light is used, that the light entering the slit is insufficient to produce an image with the exposure used, and the rest of the graph is unobtainable.

Using the ground quartz discs experiments were made with specimens of

epidermis from two other people. These specimens (Fig. 3, *a* and *b*) were thinner than those used in the above experiment being 0.065 mm. and 0.05 mm. in thickness respectively. The values for $\log \frac{J}{J_0}$ were therefore lower and readings were obtained for radiations of the wave-length 246 $\mu\mu$ with both specimens and for wave-length 240 $\mu\mu$ for one (Fig. 3 *b*). These

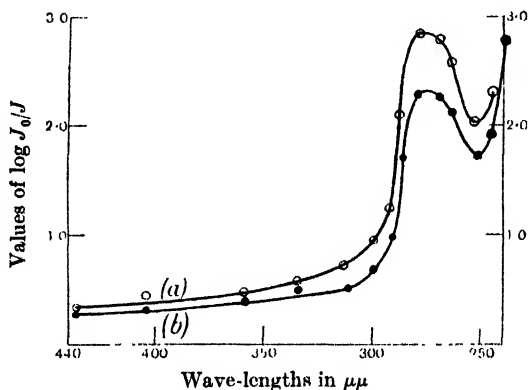


Fig. 3. Absorption curves of epidermis from two different individuals, using scattered incident light.

a. Thickness 0.065 mm.

b. Thickness 0.05 mm.

graphs resemble in shape the absorption curves of the amino-acids tryptophan and tyrosine, shown in Fig. 7 [Ward, 1923], of serum-albumin [Smith, 1929] and of caseinogen determined by myself (see Fig. 5).

Tyrosine has been found in epidermis by Buchtala [1912] and tryptophan, though not actually shown to be present in epidermis, has been found in epidermal structures such as wool to the extent of 1.8 to 1.87 % [Marston, 1928].

2. *Diminution of error due to scattering of light by employing clarified specimens of epidermis.*

So far, it has been shown that the apparent opacity of epidermis to ultra-violet light may be diminished nearly a hundredfold by altering the optical conditions of the experiments, but even the method of employing previously scattered light by no means eliminates the disturbing effects of subsequent scattering in the epidermis. Recourse was therefore had to the method of clarification, by immersing the epidermis in some liquid the refractive index of which approached that of the materials of which epidermis is composed.

The choice of clarifying agents was governed by two conditions. The reagents should not alter the chemical composition of the epidermis, nor have of themselves an absorption of ultra-violet light in the region under investigation. For this reason the ordinary clarifying agents of microscopy, xylene, toluene, chloroform, etc., were unsuitable. The reagents chosen were glycerol and glacial acetic acid, both of which fulfilled the necessary conditions. Of

these glacial acetic acid was the better, judging by visible standards. The following experiments showed that these reagents did not permanently change the chemical constitution of the epidermis.

A piece of the same epidermis as was used for the experiment shown in Fig. 2 was placed in tap-water and a photograph taken of the transmitted spectrum using parallel light. The results are shown in Table II, col. 1 and

Table II.

Wave-length in $\mu\mu$	Values of $\log J_0/J$						Percentage of incident light transmitted according to Hasselbalch	
	1	2	3	2'	3'	4	Percentage of incident light trans- mitted in Exp. 4	Calcu- lated for epidermis 0.04 mm. thick 0.08 mm. thick
437	1.78	0.79	1.70	0.40	1.82	0.02	96	—
404	1.95	0.80	1.87	0.40	1.94	0.04	91	81 65
360	2.23	1.00	2.08	0.52	2.18	0.10	81	68 46
334	2.75	1.20	2.57	0.60	2.62	0.19	65	54 29
313	3.10	1.34	3.00	0.71	2.90	0.26	55	46 21
300	3.52	1.61	3.24	0.84	3.19	0.35	44	32 10
294	3.86	2.03	3.54	0.94	3.40	0.58	26	20 4
289	—	3.12	—	1.40	—	1.54	2.9	3 0.09
280	—	3.60	—	1.78	—	1.78	1.7	—
270	—	3.05	—	1.81	—	1.83	2.0	—
265	—	3.13	—	1.61	—	1.60	2.6	—
254	—	3.33	—	1.56	—	1.34	4.6	—
246	—	—	—	1.72	—	1.43	1.5	—
240	—	—	—	2.71	—	—	—	—
238	—	—	—	3.16	—	—	—	—

- (1) Epidermis, thickness 0.08 mm. in tap-water, light parallel.
 (2) " " " cleared in glycerol, light parallel.
 (3) " " " washed and replaced in tap-water, light parallel.
 (2') " " " cleared in glacial acetic acid, light parallel.
 (3') " " " washed and replaced in tap-water, light parallel.
 (4) " " " cleared in acetic acid, incident light scattered with
 ground quartz discs.

in graphic form in Fig. 4, curve 1. The epidermis was then placed in glycerol for about an hour after which no further clearing could be observed. It was then clamped between the quartz discs and a similar series of photographs taken. The values of $\log \frac{J_0}{J}$ are shown in Table II, col. 2 and in graph form in Fig. 4, curve 2. The glycerol was then washed out and the epidermis replaced in tap-water. The series of photographs obtained with this material provided measurements of $\log \frac{J_0}{J}$ reproduced in Table II, col. 3 and in Fig. 4, curve 3, which coincides almost exactly with curve 1. The epidermis when cleared with acetic acid furnished data to produce the figures of Table II, col. 2' and the curve 2', Fig. 4. Once more it was replaced in tap-water and from the figures obtained in this experiment, the curve 3' Fig. 4, was set out and found to be almost identical with curves 1 and 3, showing that the clearing reagents did not permanently alter the constitution of the epidermis.

Finally the epidermis was placed in ether to remove any possible fat and

then cleared in acetic acid, and a series of photographs were taken with the ground quartz discs in position, with the idea of still further reducing any possible scattering. The values for $\log \frac{J_0}{J}$ obtained in this experiment are shown in Table II, col. 4, and the corresponding curve 4 in Fig. 4. In accordance with the naked eye appearance acetic acid proved a better clarifying agent than glycerol for ultra-violet light, for the less degree of absorption measured is due no doubt to a less degree of scattering.

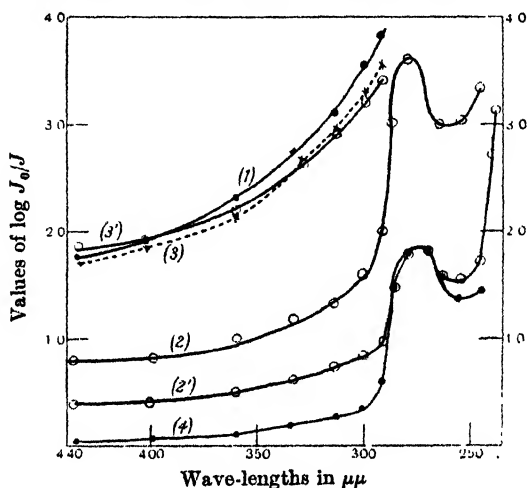


Fig. 4. Absorption curve from epidermis of the same individual as in Fig. 2. Thickness 0.08 mm.

Curve 1.	Epidermis in tap-water.	Light parallel.
" 2.	" glycerol.	Light parallel.
" 3.	" tap-water.	Light parallel.
" 2'.	" glacial acetic acid.	Light parallel.
" 3'.	" tap-water.	Light parallel.
" 4.	" glacial acetic acid.	Light scattered.

In comparing in Fig. 4 the curves 2, 2' and 4, the first thing noticed is that in general shape, the curves are similar to each other, to those in Fig. 3 and to curve *a* in Fig. 2. The actual values of $\log \frac{J_0}{J}$ are not comparable in the different experiments, as the specimen of epidermis used for the tests illustrated in Fig. 4 was thicker than those used for the experiments shown in Fig. 3.

Curve 4 (Fig. 4) showed a very interesting phenomenon. The introduction of ground quartz discs reduced the apparent opacity of epidermis, even when partially cleared, to a marked extent, as far as the wave-length $294 \mu\mu$. After that the curve coincided very closely with curve 2', corresponding to epidermis cleared similarly with acetic acid, but exposed to parallel incident light. This would show that for light of wave-length $294 \mu\mu$ and shorter, the effect of true absorption is so great as to dwarf the effect due to scattering. It is probable therefore that curve 4 shows the best approximation to the true absorption of ultra-violet light by human epidermis.

With the idea of examining in detail that part of the curve in which there is true absorption, a piece of the same epidermis was photographed in water, glycerol and acetic acid in turn. In order to obtain records of the shortest wave-lengths possible, it was decided to use, in addition to the clarifying reagents and the ground quartz discs, a quartz lens placed so as to render the rays, which had previously been allowed to come haphazard from the lamp, parallel before reaching the first scattering surface of the first quartz disc. The intensity of illumination being thereby increased, the time of exposure necessary to obtain records from the shorter wave-lengths was diminished. From these observations values for $\log J_0$ were obtained, and curves were drawn to show absorption for lines of the mercury vapour spectrum corresponding to short wave-lengths hitherto unmeasured from lack of intensity of the transmitted light. This gave greater accuracy to the curves.

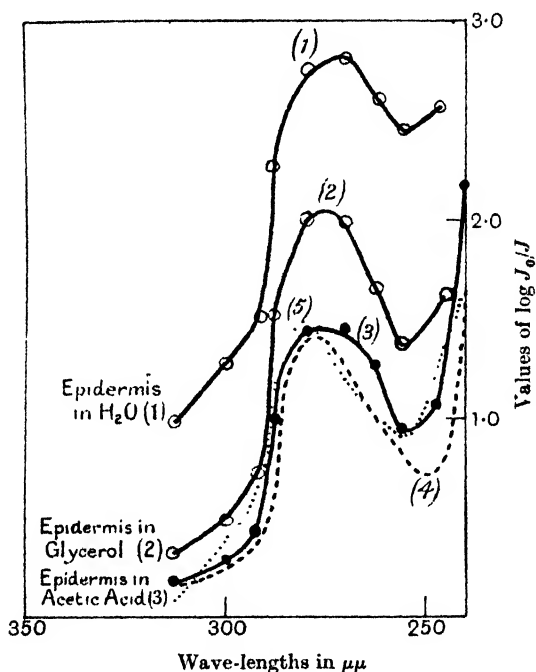


Fig. 5. Repetition of experiment on epidermis from the same individual as that used for experiments illustrated in Figs. 2 and 4, but with incident light collected on to the ground quartz discs. Thickness of epidermis 0.08 mm.

- | | |
|---------------------|---|
| Continuous Curve 1. | Epidermis in water. |
| " 2. | " glycerol. |
| " 3. | " acetic acid. |
| For comparison: | |
| Dotted Curve 4. | 2.5 % serum-albumin. Thickness of layer 1 mm. |
| " 5. | 2 % caseinogen. Thickness of layer 1 mm. |

The curve of absorption by epidermis cleared with glacial acetic acid (Fig. 5, curve 3) showed great similarity to the absorption curve of a 2.5 %

solution of serum-albumin or 2 % caseinogen solution, except that for serum-albumin the drop in the region of wave-lengths $270\mu\mu$ to $250\mu\mu$ is not so great.

Damp epidermis freed from adherent moisture by pressing between filter-paper contains 40.4 % of water, and the absorption curve illustrated in Fig. 5 is not unlike that of a similar thickness of a 2 % solution of caseinogen both qualitatively and quantitatively. The curves shown in Fig. 7 indicate that the

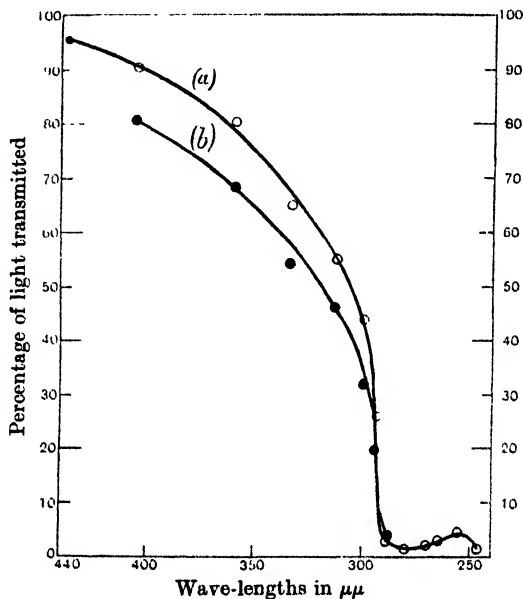


Fig. 6. Curves showing percentage transmission of light through epidermis:

a. Curve derived from Exp. 4. Epidermis 0.08 mm. thick.

b. Curve derived from Hasselbalch's experiment with epidermis 0.04 mm. thick.

opacity of epidermis to ultra-violet rays between the wave-lengths $313\mu\mu$ and $240\mu\mu$ would appear to be principally due to the content of tyrosine and tryptophan, and to be of the same order as that of the proteins, albumin and caseinogen. Cystine does not appear to be responsible for any material part of the opacity of epidermis to ultra-violet rays in the region under consideration, because the great rise in its absorption does not begin until the wave-length $225\mu\mu$.

The best approximation to the true absorption of ultra-violet light by epidermis would appear to have been obtained in Exp. 4 (Table II; curve 4, Fig. 4). Translating the logarithmic figures into arithmetical numbers the percentage of incident light which penetrates and is transmitted through the epidermis and reaches the blood-stream is shown in Fig. 6, curve (a) and in the last column but one of Table II.

It appears then that in the range of wave-lengths of the sun's light arriving at the earth's surface which are active in the cure of rickets amounts varying from 55 % for wave-length $313\mu\mu$ to 26 % for wave-length $294\mu\mu$ may be transmitted to the dermal capillaries.

For comparison a curve illustrating Hasselbalch's experiment with epidermis is included in Fig. 6 (curve b). The epidermis which he used was 0.04 mm. thick, whereas the epidermis which provided the material in the present work was 0.08 mm. thick. Nevertheless with the much thicker epidermis the proportion of transmitted light recorded for wave-lengths $404\mu\mu$ to $289\mu\mu$ was greater than that estimated by Hasselbalch. On the basis of his figures the proportion of light transmitted through epidermis of 0.08 mm.

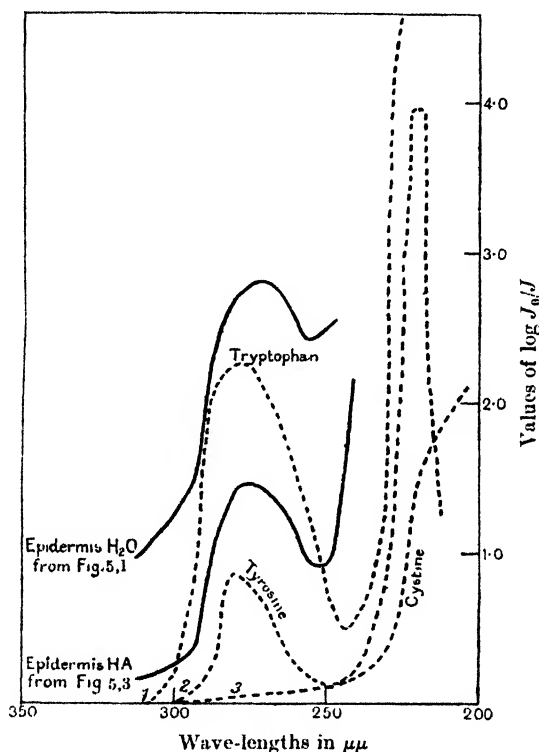


Fig. 7. Absorption curves.

- | | |
|-----------------------|----------------|
| 1. Epidermis in water | } from Fig. 5. |
| 2. " " acetic acid | |
| 3. Tryptophan. | |
| 4. Tyrosine. | |
| 5. Cystine. | |

thickness has been calculated (see Table II last column). These figures compared with those obtained in the present work show human epidermis to have from 1.5 to 30 times the transparency found by him for radiations of wave-lengths $404\mu\mu$ to $289\mu\mu$ respectively. The reason for this discrepancy is probably due to a failure on his part to differentiate between the loss of transmitted light by scattering, which loss is progressively greater as the wave-length decreases, and the loss by true absorption. It must be emphasised that much more is lost to the spectrograph by scattering when skin is irradiated

under the condition, of these experiments, than would be lost to the animal during irradiation.

SUMMARY.

1. The apparent absorption of ultra-violet light by epidermis as determined by the usual methods of photometry is not entirely due to true absorption, but is in part due to scattering of the incident light by the epidermis, which is not optically homogeneous.

2. This scattering while causing great diminution of the intensity of the light received by the spectrograph causes much less interference in transmission of the light to the underlying tissues when skin is irradiated *in vivo*.

3. For light of wave-length $300\mu\mu$ and less the effect of scattering is dwarfed by true absorption by the epidermis.

4. In shape these absorption curves resemble those of many proteins and amino-acids, *e.g.* serum-albumin, caseinogen, tryptophan and tyrosine.

5. The percentage of ultra-violet light of physiologically active wave-lengths transmitted through the epidermis is higher than previously recorded by Hasselbalch, and is calculated to be about 1.5 to 30 times greater for wave-lengths from $404\mu\mu$ to $289\mu\mu$ respectively.

My thanks are due to the Lister Institute of Preventive Medicine for facilities in carrying out these experiments, and to Sir Charles Martin for his great help, without which the work could never have been completed.

REFERENCES.

- Anderson and Macht (1928, 1). *J. Amer. Med. Assoc.* **90**, 161.
— — (1928, 2). *Amer. J. Physiol.* **86**, 320.
Bachem (1928). *J. Amer. Med. Assoc.* **90**, 563.
Buchtala (1912). *Z. physiol. Chem.* **78**, 55.
Hasselbalch (1911). *Scand. Arch. Physiol.* **25**, 55.
Hess, Weinstock and Helman (1925). *J. Biol. Chem.* **63**, 305.
Hill (1928). *J. Amer. Med. Assoc.* **90**, 1310.
Hume, Lucas and Smith (1927). *Biochem. J.* **21**, 362.
Marston (1928). *The chemical composition of wool. Council for Sci. and Ind. Research of Australia, Bulletin* 38.
Rosenheim and Webster (1927). *Biochem. J.* **21**, 389.
Smith (1929). *Proc. Roy. Soc. Lond. B* **104**, 198.
Ward (1923). *Biochem. J.* **17**, 898.
Windaus and Hess (1927). *Proc. Soc. Exp. Biol. Med.* **24**, 461.

X. THE RELATION BETWEEN CYSTINE YIELD AND TOTAL SULPHUR IN KEMP AND OUTER-COAT ANIMAL FIBRES.

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(Received December 13th, 1930.)

IN previous publications [Rimington, 1929, 1 and 2] it has been shown that practically the entire sulphur of purified wool and animal hair can be accounted for as cystine, the latter being determined colorimetrically and its quantity compared with the total sulphur of an aliquot portion of the hydrolysate. Several different varieties of wool and of hair were examined with identical results. It was noted, however, in the case of some animal hairs, more particularly of a sample of camel hair, examined, that the agreement between sulphur and cystine values was less close than in the case of true wool fibres. Such a difference might possibly be explained on the ground that the medullary cells of the hair shaft contain sulphur compounds other than cystine. Medulla is present in most animal hairs; it is absent however from true wool fibres.

In order to test this possibility and to complete the series of observations already reported, it was decided that an examination should be made of some typical kemp, of lambs' birth-coat, which is also medullated, and of further samples of camel hair. The investigation was rendered difficult by the necessity of sorting each sample by hand; in some cases it took several weeks to pick out sufficient material for analysis. In the case of camel hair, and Scotch blackface wool, one is also confronted by the difficulty of classification, nearly all gradations being met with between true wool and true kemp.

EXPERIMENTAL.

Each sample, after separation, was degreased in warm benzene and washed in the manner already described [Rimington, 1929, 1]. Samples of 1 g. were taken for hydrolysis. These were boiled for 5 hours with 20 cc. of 20 % hydrochloric acid. Two portions of 5 cc. each were then withdrawn for determination of total sulphur content, which was carried out by Rimington's [1930, 1] modification of the Benedict-Denis method for the determination of sulphur in urine. Another 5 cc. sample was measured into a 50 cc. graduated flask, diluted somewhat and brought to about $N/10$ acidity by cautious addition of 20 % sodium hydroxide solution. After filling to

50 cc. and filtering, this solution (2 cc.) was employed for the colorimetric determination of cystine, Folin and Marenzi's method as amended by Rimington [1930, 2] being employed.

Since, by Folin and Marenzi's [1929] technique it is now possible to obtain a reagent free from molybdenum compounds and giving no colour with pure tyrosine, this method is to be preferred to that of Sullivan [1926], which is technically somewhat difficult and which gives only a limited proportionality between colour intensity and cystine concentration. In the present study it was not considered necessary to employ Sullivan's method at all since earlier work [Rimington, 1929, 1] had shown the absence from hirsute structures of disulphide linkages other than those due to cystine. Where determinations were made of the total sulphur of the dry material, these were carried out as described in previous publications.

The results are recorded in Table I.

Table I. *Hydrolysis of 1 g. material by 20 cc. of 20 % HCl for 5 hours.*

Material	Total S % dry wt.	Cystine in hydrolysate by Folin-Marenzi- Rimington method g.	Total S of hydrolysate (as cystine) g.	Ratio*
Camel hair (kemp-free) I	3.25	0.1100	0.1169	+ 5.90
„ kemp I	—	0.1030	0.1134	+ 9.17
„ kemp II	—	0.1013	0.1055	+ 3.98
„ (mixed sample of 6 g.)	3.25	0.5510 (Sullivan)	0.5908	+ 6.73
Welsh Mountain wool	4.08	0.1260	0.1255	- 0.40
„ kemp	—	0.0975	0.0988	+ 1.32
„ birth-coat I	3.06	0.0957	0.0954	+ 0.31
„ birth-coat II	2.72	—	—	—
Scotch blackface kemp	—	0.0787	0.0811	+ 2.96

* $\frac{\text{Total S of hydrolysate as cystine} - \text{cystine (colorimetric)}}{\text{Total S as cystine}} \times 100.$

DISCUSSION.

Only in the case of camel hair can it be said, on reference to Table I, that there is any significant difference between the cystine content of kempy material (medullated fibres) as determined directly and by calculation from the total sulphur content. The differences noted in the case of Welsh mountain wool, kemp and birth-coat are well within experimental error.

The series is somewhat limited on account of the difficulty of obtaining sufficiently large quantities of kemp from normally fine-woolled sheep. It appears however that kemp, containing a large proportion of medullary cells, resembles non-medullated sheep's wool in containing no significant quantities of any sulphur-containing amino-acid other than cystine.

Camel hair, both inner and outer coat, as previously found shows evidence of non-cystine, sulphur-containing compounds. In this connection it should

be remembered that these fibres contain a dark, reddish-brown pigment and it is not improbable that this pigment itself contains sulphur.

The total sulphur content of Welsh mountain lambs' birth-coat is very considerably less than that of the wool of the same breed, just as the "puppy coat" of rats is less rich in cystine and sulphur than the corresponding hair of the adult [Lightbody and Lewis, 1929]. Rabbits' fur also increases in sulphur content with increase in the age of the animal [Düring, 1897]. It appears, in agreement with Barritt and King [1926], that the medullary cells are less rich in cystine than are the cortical cells of the fibre.

SUMMARY.

1. Samples of Scotch blackface kemp, Welsh mountain wool, kemp and lambs' birth-coat have been examined and in all cases practically the entire sulphur could be accounted for as cystine.

2. In the case of two separate samples of camel hair, both true hair (inner coat) and coarse outer-coat fibres contained more sulphur than could be accounted for as cystine. It is suggested that the brown-red pigment present in these samples may account, in part at any rate, for the discrepancy.

The author wishes to thank Mr A. T. King for his continued interest in the subject of this work, and Mr H. Rothera for valuable help in the preparation of the material.

REFERENCES.

- Barritt and King (1926). *J. Text. Inst.* **17**, 386 T.
During (1897). *Z. physiol. Chem.* **22**, 281.
Folin and Marenzi (1929). *J. Biol. Chem.* **83**, 103.
Lightbody and Lewis (1929). *J. Biol. Chem.* **82**, 485.
Rimington (1929, 1). *Biochem. J.* **23**, 41.
— (1929, 2). *Biochem. J.* **23**, 726.
— (1930, 1). *J. Soc. Chem. Ind.* **49**, 139 T.
— (1930, 2). *Biochem. J.* **24**, 1114.
Sullivan (1926). *U.S. Public Health Rep.* **41**, 1030.

XI. COLOUR REACTIONS OF STEROLS WITH NITRIC ACID.

BY OTTO ROSENHEIM AND ROBERT KENNETH CALLOW.

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(Received December 9th, 1930.)

IN the course of experiments on the dehydrogenation of zymosterol by means of mercuric acetate, it was noticed that nitric acid, used for cleaning the reaction vessel, gave an intense blue colour reaction with fragments of the reaction mixture left in the flask. This chance observation led to a systematic study of the reaction of nitric acid with sterols. The characteristic colours produced with certain sterols were found to be somewhat transient when pure colourless nitric acid was employed. The oxidising action of the acid was retarded and the colour reactions were stabilised to a certain extent by using a mixture of glacial acetic acid with nitric acid (1:4). Finally it was found that nitric acid containing the acetates of metals (magnesium, cerium, cadmium, mercury, uranium, etc.) gave fairly stable colour reactions. They differed to some extent with different metals, and in order to simplify description the following notes are restricted to the reactions of the "mercury reagent." The reagent is prepared by dissolving 25 g. of mercuric acetate in 100 cc. of nitric acid (sp. gr. 1.42). Nitrous acid interferes with the colour reactions, and it is necessary to decolorise the solution with a few crystals of urea. When this precaution is used the reagent apparently keeps indefinitely.

The reaction is carried out by adding to a chloroform solution of the sterol an equal volume of the reagent, and shaking immediately. On account of the high specific gravity of the mercury reagent, the mixture separates rapidly into an upper coloured chloroform layer and a lower, usually colourless, layer of the reagent.

1. *Red colours* are given by *allo*-cholesterol, *allo*-sitosterol, cholesterolene, ψ -cholestene and β -cholesterol. The tint of the red colours varies somewhat with different sterols and is orange, carmine red or magenta according to concentration, but all the solutions show selective absorption in the region of 500μ . A red colour is given only by those sterols which are believed to possess a $\Delta^{1,2}$ (or $\Delta^{1,13}$) ethenoid linkage in the molecule and which have been found previously [Rosenheim, 1929] to give a red colour reaction with trichloroacetic acid. It may be assumed therefore that the mechanism of the reaction consists, as in the latter case, in the formation of coloured carbonium salts and that a red colour reaction with the mercury reagent is specific for

and dependent on the presence of a $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage in the sterol ring system.

Considerations of the relationship of colour reactions and absorption spectra of sterols to their structure have led Heilbron and Spring [1930] to the conclusion that "selective absorption of sterols is conditioned by the presence of two ethenoid linkages in the molecule, one of which must apparently be in the $\Delta^{1,13}$ (or $\Delta^{1,2}$) position." The fact that oxycholesterilene possesses two ethenoid linkages and a highly selective absorption at $280\mu\mu$ [Rosenheim and Adam, 1929], but gives a negative reaction with both the mercury reagent and trichloroacetic acid (see later) weakens the evidence adduced by Heilbron and Spring for such a generalisation.

2. *A blue colour*, following a transient pink, is given by ergosterol and its esters when the reaction is carried out with dilute solutions (5 mg. or less in 1 cc. chloroform), a marked reaction still showing with 0.01 mg. The tint is a characteristic peacock-blue, and the solution does not show any selective absorption. On prolonged standing the blue colour changes through magenta into a dull shade of red. The lower acid layer rapidly loses its initial rose-red colour and becomes colourless.

An entirely different reaction results when relatively concentrated solutions of ergosterol (10 mg. or more in 1 cc. chloroform) are used. The same initial pink colour appears on mixing with the reagent, but it rapidly changes into orange and a clear yellow, instead of into blue. The acid layer remains colourless throughout.

The latter reaction has been found useful as an index of the purity of ergosterol, since oxidised or otherwise changed specimens give finally an intense green reaction under the above conditions [Callow, 1931]. The sensitiveness with which the reaction indicates such changes is demonstrated by allowing a 1 % chloroform solution of ergosterol to stand in a stoppered cylinder exposed to diffused daylight. Within a few hours the still colourless solution gives with the mercury reagent a purple and finally an intense green colour, showing a green fluorescence. The resemblance of this reaction to that of *isoergosterol* (see later), and the observation that this change does not occur in solutions protected from light, suggests that it is due to the production of *isoergosterol*. Under the influence of light, chloroform decomposes into phosgene and hydrochloric acid, and the latter is known to isomerise ergosterol into *isoergosterol* at ordinary temperatures. The liberation of hydrochloric acid from chloroform has been shown to be the cause also of the change from red to green when a chloroform solution of bilirubin is exposed to sunlight [Thudichum, 1883].

Ergosterol peroxide [Windaus and Brunken, 1928] and dehydroergosterol peroxide [Windaus and Linser, 1928] yield greenish-blue reactions similar to that of dilute ergosterol solutions.

The reaction of *isoergosterol* [Reindel, Walter and Rauch, 1927] differs essentially from that of ergosterol. The colour changes end in olive green after

passing rapidly through red to purple and blue. In the blue stage a well defined absorption band at 550μ can be observed, and the final green stage shows a green fluorescence. A further distinction from ergosterol is seen in that the colour changes are identical in concentrated or dilute solutions. The influence of the complex Hg ion is evident in this reaction: when cadmium acetate replaces mercuric acetate, the *isoergosterol* reaction stops at the red stage. The various *isoergosterols* of Bills and Cox [1930] have not been investigated.

3. *Colourless solutions* are obtained in the reactions with (1) cholesterol and its derivatives (cholesteryl chloride, α -cholesterol oxide, α -cholestaneetriol, cholestenone, oxycholestenone, oxycholesterilene, dihydrocholesterol), (2) coprosterol and coprostanone, (3) the phytosterols: stigmasterol, sitosterol, γ -sitosterol, dihydrositosterol, (4) zymosterol (impure preparations give an intense blue-green reaction, due to ergosterol), lanosterol (from lanolin) and β -amyrol, (5) dihydroergosterol and tetrahydroergosterol.

From these facts it is apparent that only those sterols can give colour reactions with nitric acid or with the mercury reagent which possess either the $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage or the unknown ethenoid linkages of the ergosterol molecule. The non-reactivity of the two partially reduced ergosterols is not surprising, since a shifting of the double linkages may have taken place during reduction. Both still contain an "inert" ethenoid linkage, which Heilbron and Spring [1930] connect with a positive bromine reaction (Tortelli-Jaffé). It may be suggested however that the latter reaction is a secondary one and connected with the destructive action of bromine on the ergosterol molecule. In favour of this view is the fact, found by us, that iodine replacing bromine in the Tortelli-Jaffé reaction, gives a green colour with ergosterol, but does not react with either dihydroergosterol or tetrahydroergosterol.

Other reactions. (1) *Vitamin A and carotene.* Most cod-liver oils give with the mercury reagent a series of indefinite colour reactions ending in red. Other liver oils, which are many times as rich in vitamin A as cod-liver oil [Rosenheim and Webster, 1927], such as certain mammalian liver oils, salmon-liver oil, etc., give a brilliant transient blue colour with the reagent. The unsaponifiable fraction of these oils, and also of cod-liver oil, yields an intense and rather more stable blue colour, which is apparently identical with the colour produced with the unsaponifiable fraction by AsCl_3 , SbCl_3 and trichloroacetic acid [Rosenheim and Webster, 1926]. The relationship of these colour reactions to vitamin A remains still to be defined.

In view of the resemblance of the colour reactions of cod-liver oil to those of carotene, which originally suggested a relationship of the latter to vitamin A [Rosenheim and Drummond, 1925] it was not surprising to find that carotene also gives a blue colour reaction with the mercury reagent. In distinction from the trichloroacetic acid reaction of carotene, the reaction is a transient one.

(2) *Vitamin D.* Irradiated ergosterol, freed from unchanged ergosterol

by digitonin, gives a transient red colour, followed by a permanent olive green with both the mercury reagent and trichloroacetic acid. Since the same reaction is given by biologically inactive, over-irradiated or oxidised specimens, it is probably due to one or more of the inactive substances produced from ergosterol by irradiation.

(3) *Bile acids and their derivatives.* Desoxycholic acid and cholic acid possess the same ring system as the saturated sterols and were accordingly found to be non-reactive. On distillation they form, by loss of water, the unsaturated choladienic and cholatrienic acids [Wieland and Weil, 1912], specimens of which we owe to the kindness of Professor Wieland. Whilst choladienic acid is non-reactive, cholatrienic acid in dilute chloroform solution gives with the mercury reagent a gentian-blue colour reaction, the lower acid layer turning deep red. An intense blue colour slowly develops with trichloroacetic acid on standing for a few hours, the blue solutions in both reactions showing a band in the red region of the spectrum with a maximum at $650\mu\mu$. It seems therefore that the ethenoid linkages connected with C_3 and C_7 , which are common to both acids, are unable to react, and that the colour reaction of cholatrienic acid depends on the presence of the $\Delta^{11,12}$ or $\Delta^{12,13}$ linkage.

(4) "*Oxycholesterol*," the mixture of substances obtained by debromination of cholesterol dibromide with sodium acetate [Lifschutz, 1919] or by treatment of cholesterol with benzoyl peroxide, gives with the mercury reagent an immediate gentian-blue colour, showing the characteristic "oxycholesterol" absorption band at $650-680\mu\mu$. Traces of this product, sufficient to be detected by the reagent, are formed from cholesterol under all conditions where oxidation may take place. A positive reaction is given by cholesterol after recrystallisation in the presence of charcoal, after irradiation in air or after heating to the melting point in air, etc. Since "oxycholesterol" gives an identical reaction also with trichloroacetic acid, $AsCl_3$ or $SbCl_3$ [Rosenheim, 1927, 1929], and thus behaves like an unsaturated sterol, it may be suggested that the substance contains an additional ethenoid linkage, and is formed by dehydrogenation of cholesterol. Such a suggestion is strengthened by the ease with which oxidising agents like perbenzoic acid dehydrogenate sterols [Windaus and Lüttringhaus, 1930].

SUMMARY.

1. Nitric acid gives characteristic colour reactions with certain sterols. A solution of mercuric acetate in nitric acid ("mercury reagent") is recommended as a general reagent.

2. A red colour with the mercury reagent indicates the presence of the $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage in sterols.

3. A greenish-blue colour is given by ergosterol in dilute solutions, whilst a yellow colour results when concentrated solutions of ergosterol are employed. The latter reaction serves as a useful index for the purity of ergosterol.

4. A gentian-blue colour is given by products containing vitamin A (certain liver oils and the "unsaponifiable" of cod-liver oil).

REFERENCES.

- Bills and Cox (1930). *J. Biol. Chem.* **84**, 455.
Callow (1931). *Biochem. J.* **25**, 87.
Heilbron and Spring (1930). *Biochem. J.* **24**, 133.
Lifschütz (1919). *Z. physiol. Chem.* **106**, 279.
Reindel, Walter and Rauch (1927). *Liebig's Ann.* **452**, 34.
Rosenheim (1927). *Biochem. J.* **21**, 386.
—— (1929). *Biochem. J.* **23**, 49.
—— and Adam (1929). *Proc. Roy. Soc. Lond. B* **105**, 422.
—— and Drummond (1925). *Biochem. J.* **19**, 753.
—— and Webster (1926). *Biochem. J.* **20**, 1342.
—— — (1927). *Nature* **120**, 440.
Thudichum (1883). *Moleschott's Beitr.* **13**, 327.
Wieland and Weil (1912). *Z. physiol. Chem.* **80**, 287.
Windaus and Brunken (1928). *Liebig's Ann.* **460**, 225.
—— and Linsert (1928). *Liebig's Ann.* **465**, 148.
—— and Lüttringhaus (1930). *Liebig's Ann.* **481**, 119.

XII. THE PURIFICATION OF ERGOSTEROL.

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(Received December 18th, 1930.)

INVESTIGATIONS proceeding in this laboratory on the formation of vitamin D made it necessary to obtain ergosterol in as pure a condition as possible. Great variations are recorded in the literature in the physical constants of ergosterol which has been the starting material for chemical and biochemical investigations, and it was considered essential to check the criteria of purity put forward by Tanret [1908] and subsequent workers. Although ergosterol of a high degree of purity may be obtained from certain specimens of crude yeast ergosterol by recrystallisation from a mixture of alcohol and benzene (2:1), as described by Bills and Honeywell [1928], the use of this solvent does not invariably yield products with equally high specific rotation [*cf.* Bills and Cox, 1929; Heilbron, Sexton and Spring, 1929]. According to the experience gained in this laboratory, recrystallisation from alcohol-benzene is extremely useful for the removal of zymosterol from ergosterol, but fails to free it from α -dihydroergosterol. α -Dihydroergosterol appears to be a normal constituent of the sterol mixture occurring in yeast [Callow, 1930, 1931], and its presence in varying proportions accounts almost entirely for the differences in the physical constants of ergosterol recorded in the literature. It is however removed by the method of fractional recrystallisation of the benzoates described here, and ergosterol from different sources, both British and Continental, has yielded purified specimens with no significant differences in their physical properties. No support is therefore given to the conclusion of Bills and Cox [1929] and Bills and McDonald [1930] that natural isomerides of ergosterol separable only with difficulty commonly occur in specimens of different specific rotation obtained from yeast grown under different conditions.

In the course of the preliminary investigations, ergosterol was distilled under reduced pressure. The conclusion of Reindel and Detzel [1929] that this is useless as a method of purification was confirmed.

Purification by means of the recrystallisation of an ester was then considered. The acetate [Windaus and Grosskopf, 1922] and the isobutyrate [Bills and Honeywell, 1928] have previously been used for this purpose. Preliminary experiments with ergosteryl ethyl carbonate, prepared from ergosterol by the action of excess of ethyl chloroformate in pyridine solution, indicated that this ester was not a suitable one. The benzoate, however, which has the advantage of being prepared under mild conditions of reaction, gave

an immediate separation into fractions of different specific rotation. Benzoylation of different specimens of yeast ergosterol was carried out with benzoyl chloride in pyridine under uniform conditions, as described in detail below, and the benzoates, which underwent an initial fractionation in the process of separation, were then further fractionally recrystallised from ethyl acetate. Purification was rapid when fractions were collected at 37°, and the head fractions attained a constant specific rotation after five or six recrystallisations at this temperature. The sterol was obtained from the purified benzoate by hydrolysis with alcoholic potassium hydroxide, and freed from traces of decomposition products by recrystallisation.

Three crystalline forms of ergosteryl benzoate were observed. It usually crystallises as a bulky mass of fine needles, a form which is actually stable only at high temperatures. The modification stable at ordinary temperatures forms rectangular plates. Rapidly cooled solutions deposit leaflets belonging to the monoclinic system, which soon pass into a mixture of the other two forms.

The specific rotations of the specimens of purified ergosteryl benzoate and ergosterol were concordant within the limits of the probable errors of determination. In Table I are given the values obtained, together with the melting-points and the figures recorded in the literature.

Table I.

M.P.	Ergosterol [α] in chloroform		M.P.	Ergosteryl benzoate [α] in chloroform		Observer
	Anhydrous	Hydrated				
165°	[α] _D - 132°	[α] _D - 126°	—	—	—	Tanret [1908]
166–183°	[α] ₅₄₆₁ ²⁰ - 170.6°	—	164–168°	[α] _D ²⁵ - 177°*	—	Bills and Honeywell [1928]
	[α] _D ²⁰ - 132°	—				
	—	—				
160–161°	[α] _D ²⁰ - 133.1°	—	168–170°	[α] _D ²³ - 68°	—	Windaus and Rygh [1928]
			168–170°	[α] _D ²³ - 71°	—	Wieland and Asano [1929]
160–163°	[α] ₅₄₆₁ ²⁰ - 174.2°	[α] ₅₄₆₁ ²⁰ - 167.2°	169–171.5°	[α] ₅₄₆₁ ²⁰ - 88.3°	—	Callow
	[α] _D ²⁰ - 135°	[α] _D ²⁰ - 128.7°				

* This exceptional value is not a misprint, as suggested by Windaus and Rygh [1928]; cf. Bills and McDonald [1930].

The values of the specific rotation now recorded are slightly higher than those found originally by Tanret [1908] for ergosterol from ergot. The molecule of water of crystallisation in ergosterol crystallised from aqueous alcohol is difficult to remove completely without decomposition. The specific rotation of the hydrated sterol which has been kept over calcium chloride for 3–4 days is the most reliable index of its purity, accompanied by a confirmatory determination of the loss of weight on heating. Prolonged storage of ergosterol over a drying agent causes dehydration which is followed by rapid oxidation in air. In sealed tubes it is unchanged for long periods.

EXPERIMENTAL.

Material.

The following data were obtained for the samples of yeast ergosterol used for this work: (A) M.P. 160–162°, $[\alpha]_{5461}^{20}$ – 156·9°; (B) M.P. 159–162°, $[\alpha]_{5461}^{20}$ – 157·7°; (C) M.P. 160–163°, $[\alpha]_{5461}^{20}$ – 156·6°; (D) M.P. 160–163°, $[\alpha]_{5461}^{20}$ – 158·4°; (E) M.P. 160–163°, $[\alpha]_{5461}^{20}$ – 161·2°; (F) M.P. 155–160·5°, $[\alpha]_{5461}^{20}$ – 137·2°. The specimens were from four different sources, and all except the last had been recrystallised from alcohol-benzene at least once. The melting-points were determined in sealed capillary tubes. All specific rotations recorded here were measured in chloroform containing alcohol (B.P.) in a concentration of about 1 % in a 4 dm. tube, the solutions being generally made up at 20° and measured at the same temperature with an instrument reading to 0·01°. Even purified ergosterol melts over a range of temperature in a capillary tube, and the melting-point is a less reliable and sensitive index of purity than the specific rotation.

One specimen of ergosterol (A), distilled from a flask in a metal-bath at 260–270°/0·1–1·0 mm., gave a pale yellow distillate, $[\alpha]_{5461}^{20}$ – 145·3°. A purified sample of ergosterol, $[\alpha]_{5461}^{20}$ – 164°, began to distil at 192°/0·001–0·01 mm., and the bulk passed over at 198° (bath at 250–260°). A sublimate began to come off at an appreciable rate at a bath temperature of 180°. The distillate was white, and had M.P. 158–161·5°, $[\alpha]_{5461}^{20}$ – 164·1°; the specific rotation had therefore fallen, since the distillate was anhydrous. Recrystallised from alcohol-benzene (2:1) it had $[\alpha]_{5461}^{20}$ – 161·2°.

Another specimen of ergosterol (F) was fractionally sublimed under the conditions used for distillation of vitamin D [Askew *et al.*, 1930]. Fractions were obtained from 0·5 g. at 135° (0·062 g., M.P. 160–164°, $[\alpha]_{5461}^{20}$ – 135°), at 145° (0·044 g., M.P. 156–163°), at 155° (0·317 g., M.P. 156·5–163°, $[\alpha]_{5461}^{20}$ – 148°), and a residue was left (0·06 g., M.P. 154·5–161·5°). These results did not justify further work.

The distilled materials were hygroscopic and very readily oxidised in air. Over calcium chloride in a desiccator, kept in a cupboard away from bright light, and opened at intervals, weighed samples turned yellow and gained in weight owing to absorption of oxygen. Reindel and Detzel [1929] made a similar observation, but, contrary to their statement, no break was observed in the curve of increase in weight at a composition corresponding to a “labile moloxide,” $C_{27}H_{42}O_3$. The absorption of oxygen, after a perceptible initial acceleration, went on at a continuously decreasing rate until practical constancy of weight was attained after a year at an increase of 19·5–21 %, corresponding to the addition of five atoms of oxygen (calculated, 20·9 %). The measurements are illustrated by the curve in Fig. 1. It seems probable that this rapid oxidation is due to the combined effects of autocatalysis, fine state of division, and complete initial dehydration. Samples of recrystallised ergosterol kept under the same conditions show a slight initial loss of weight followed, after two or three months, by an increase in weight, the rate of

which at first increases and then decreases, accompanied by a deepening yellow colour. One sample, after a year, gained 18 % in weight, and was still gaining. It appeared likely to reach the same limit as the distilled products

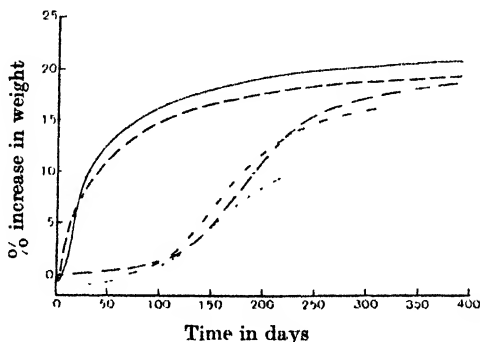


Fig. 1. Increase in weight of ergosterol kept over CaCl_2 in air.

- Distilled, purified ergosterol. - - - Distilled ergosterol (A).
 - - - - - Ergosterol (E).
 - . - . - Ergosterol (A), recrystallised from alcohol-benzene.
 Ergosterol purified *via* the benzoate.

after a further six months. The nature of the products formed has not yet been investigated.

Ergosteryl ethyl carbonate. Ergosterol (3.4 g.) was dissolved in dry pyridine (60 cc.) and ethyl chloroformate (14 cc.) was added slowly with constant shaking and cooling. After standing for 30 minutes the mixture was poured into water and the precipitated solid was collected and washed. Much frothing occurred in both stages of the preparation. Recrystallised from absolute alcohol and from ethyl acetate, *ergosteryl ethyl carbonate* was obtained as thin plates, m.p. $150\text{--}153.5^\circ$, $[\alpha]_{\text{D}}^{20} - 111.1^\circ$. The yield was 2 g. (Found (micro): C, 79.3; H, 10.4 %. $\text{C}_{30}\text{H}_{46}\text{O}_3$ requires C, 79.3; H, 10.1 %.) The ultra-violet absorption spectrum in alcoholic solution closely resembled that of ergosterol. When the carbonate was irradiated in solution under the same conditions as ergosterol, the product showed equal antirachitic activity. The ester, in this respect, thus resembles the acetate.

A small fraction of the crude product was insoluble in alcohol, and crystallised from ethyl acetate in needles, m.p. $206\text{--}212^\circ$. Fractionation of the alcoholic mother-liquors showed the presence of unchanged ergosterol. Hydrolysis of the ester with alcoholic potassium hydroxide yielded ergosterol, which, recrystallised from 95 % alcohol, had m.p. $159\text{--}162^\circ$, $[\alpha]_{\text{D}}^{20} - 164.2^\circ$. No further work was done with the *ergosteryl ethyl carbonate*, since the conditions of its preparation were less satisfactory than those of the benzoate, and it had no advantage with respect to purification.

Preparation of ergosteryl benzoate. Ergosterol (1 part) in dry pyridine (20 volumes) was treated with redistilled benzoyl chloride (2.5–3 volumes) in small portions while the flask was shaken and cooled under the tap. The

development of a permanent pink colour was a reliable indication of the presence of a slight excess of benzoyl chloride. It was necessary to use much more than the calculated quantity of benzoyl chloride in order to obtain complete esterification, and the excess required varied considerably with different specimens, probably according to the amount of solvent of crystallisation or the degree of dryness of the pyridine. Under these conditions part of the benzoate crystallised out directly from the reaction mixture, together with pyridine hydrochloride. The necessity of pouring the whole into water was obviated, and a fractionation was obtained. After cooling to room temperature, the solid which separated (fraction I) was collected and washed with a very little pyridine, and the filtrate was cooled to -4° . Fraction I was, in the meantime, washed thoroughly with water and finally with alcohol. Fraction II, obtained at -4° , was treated similarly, and the aqueous washings from the two crops (in all 40 volumes) were added to the filtrate. The precipitate thus produced (fraction III) was collected and washed. Ethyl benzoate, in some cases in large amount, separated with the precipitate. In the light of subsequent work, it is evident that this was derived from the alcohol of crystallisation of the α -dihydroergosterol originally present. Pyridine was recovered from the filtrate.

In a typical preparation sample D of ergosterol (45 g.) gave the following fractions: (I) 35.8 g., m.p. $164-171^{\circ}$, $[\alpha]_{5461}^{20} = 86.8^{\circ}$; (II) 7.1 g., m.p. $163-168.5^{\circ}$, $[\alpha]_{5461}^{20} = 82.9^{\circ}$; (III) 7.0 g., m.p. $135-150^{\circ}$, $[\alpha]_{5461}^{20} = 36^{\circ}$; total yield, 91 %.

Purification of ergosteryl benzoate. In general, fractions I and II were worked up in the same scheme of fractional crystallisation, whilst fraction III was treated separately for the isolation of admixed sterols whose presence was suspected. The system ultimately adopted was to recrystallise fraction I repeatedly until no further alteration in specific rotation took place, and to utilise the mother-liquors for the recrystallisation of subsequent batches of crude benzoate. In this way there accumulated mother-liquors in which impurities of the original ergosterol collected. The ethyl acetate used as solvent was freed from acetic acid and higher esters and dried. The crude benzoate was dissolved, in a current of nitrogen, in 25-35 volumes of boiling ethyl acetate to give a nearly saturated solution, which was then allowed to cool in a closed flask to 37° in the hot room. It was found in the first stages of the work that crystallisation at 37° rather than at room temperature gave a much more efficient purification, and much reduced the number of recrystallisations necessary. Oxidation took place to an undesirable extent in the mother-liquors if hot solutions were exposed freely to the air. A mixture of butyl alcohol and benzene (2:1), and 80 % aqueous pyridine were also tried as solvents, but had the disadvantage of being difficult to remove and of hindering the recovery of material from mother-liquors. Pyridine seemed particularly to favour decomposition and oxidation. Ethylene dichloride was unsuitable because of the difficulty of keeping it free from traces of hydrogen chloride,

to which ergosterol is very sensitive. None of these solvents, moreover, gave evidence of further purification after exhaustive crystallisation from ethyl acetate.

The course of the purification of the benzoate from sample F may be taken as an example. Fraction I, twice recrystallised from ethyl acetate at 37° yielded material (1), $[\alpha]_{5461}^{20} - 83.7^\circ$. Crystallisation of this (33 g.) from ethyl acetate (1100 cc.) yielded (2), $[\alpha]_{5461}^{20} - 86.2^\circ$ (20 g.), and so, in succession, (3), $[\alpha]_{5461}^{20} - 86.7^\circ$ (10.8 g.); (4), $[\alpha]_{5461}^{20} - 87.2^\circ$ (6.4 g.); (5), $[\alpha]_{5461}^{20} - 88.1^\circ$ (4 g.); (6), $[\alpha]_{5461}^{20} - 88.0^\circ$ (2.8 g.). In another case (sample D above), the successive fractions were: (1) M.P. 167–172°, $[\alpha]_{5461}^{20} - 87.6^\circ$; (2) (from butyl alcohol-benzene) M.P. 167–171.5°, $[\alpha]_{5461}^{20} - 88.0^\circ$; (3) M.P. 166–171.5°, $[\alpha]_{5461}^{20} - 88.5^\circ$; (4) (from butyl alcohol-benzene) M.P. 169–171.5°, $[\alpha]_{5461}^{20} - 88.7^\circ$. A third specimen of benzoate (from samples B and C), after purification, had $[\alpha]_{5461}^{20} - 88.0^\circ$, and then, in two successive recrystallisations from ethyl acetate, gave material having $[\alpha]_{5461}^{20} - 88.15^\circ$, and $[\alpha]_{5461}^{20} - 88.2^\circ$. Comparable figures were obtained in all cases for the benzoate recovered from the mother-liquors of the head fractions, separated in later stages of the fractionation. Thus, after five or six recrystallisations from ethyl acetate, a pure, homogeneous benzoate, having a mean specific rotation $[\alpha]_{5461}^{20} - 88.3^\circ$, without significant variation, was obtained from all the samples of ergosterol.

Rectangular plates accompanied the usual needles of ergosteryl benzoate, as recorded by Wieland and Asano [1929], most frequently when the mother-liquors of fractions collected at 37° were allowed to stand at room temperature. It was ultimately discovered that from butyl alcohol-benzene solutions, the separation of either plates or needles could be obtained by seeding with the required form, although rapid cooling favoured the separation of plates, and slow cooling the separation of needles. Both forms were obtained from the purified benzoate with the same specific rotation, $[\alpha]_{5461}^{20} - 88.4^\circ$. No attempt was made to determine the transition point of the two forms, but the slow replacement of needles by plates in the presence of various solvents shows that the plate form is the stable one at room temperatures. A specimen of plates began to melt at 165° to give a cloudy mixture, in which a mass of fine needles could be discerned with the aid of a lens, and became clear at 170°, so that the needle form is evidently the stable one at high temperatures. A third crystalline form was observed when solutions of the benzoate were cooled rapidly without agitation. Leaflets separated which were replaced by a felted mass of needles and a few plates when the mixture was stirred. Isolation of the leaflets in bulk was impossible, but in portions of the solution in butyl alcohol-benzene transferred to a microscope slide it was possible to observe hexagonal leaflets, belonging to the monoclinic system, which dissolved and were replaced by rectangular plates and a few needles, the latter afterwards gradually dissolving.

Ultra-violet irradiation of ergosteryl benzoate gave a product with about 1/8 the antirachitic activity of ergosterol similarly treated, but the activity

rose to that of the irradiated free sterol when the product was hydrolysed [cf. Windaus and Rygh, 1928].

Hydrolysis of ergosteryl benzoate. This was done by heating the benzoate with 25 parts of 3 % alcoholic potassium hydroxide and boiling for 5 minutes after solution was complete. As a precaution against oxidation, the reaction was carried out in an atmosphere of nitrogen. The ergosterol which separated on cooling was collected, washed with alcohol and water, recrystallised from 40–50 parts of 95 % alcohol, and dried for several days over calcium chloride. The yield was about 67 %, and a further 15 %, generally of slightly lower rotation, could be obtained by working up the mother-liquors.

Properties of purified ergosterol. The characteristics of the pure ergosterol thus obtained from different samples were as follows:

(A) M.P. 160–162°, $[\alpha]_{5461}^{15} - 165^\circ$, loss at 100°/12 mm., 4.5 % (calculated for $C_{27}H_{42}O$, H_2O : 4.5 %);

(B + C) M.P. 160–163°, $[\alpha]_{5461}^{20} - 166.8^\circ$, $- 167.7^\circ$, $- 167.2^\circ$ (different batches; mean, $- 167.2^\circ$), $[\alpha]_D^{20} - 128.7^\circ$, anhydrous substance, $[\alpha]_{5461}^{20} - 174.2^\circ$, $[\alpha]_D^{20} - 135^\circ$, loss at 120°/0.1 mm., 4.5 %;

(D) M.P. 160–163°, $[\alpha]_{5461}^{20} - 166.5^\circ$, loss at 120°/10 mm., 4.7 %;

(F) M.P. 160–163°, $[\alpha]_{5461}^{20} - 165.9^\circ$.

Dehydration of ergosterol by heating under reduced pressure in an Abderhalden "pistol" with steam or xylene vapour was accompanied by slight decomposition, and the specific rotation was a little below the calculated value. The material was generally slightly sintered and had acquired a yellow tinge. Less decomposition occurred when the ergosterol was quickly heated to just above its melting-point in a current of pure, dry nitrogen. The anhydrous material gained in weight rapidly, owing to oxidation, when exposed to the air. Purified ergosterol gained weight in a similar way to unpurified ergosterol when kept over calcium chloride (see Fig. 1). No change in specific rotation or melting-point was found after six months in a specimen kept in the dark in a sealed, evacuated tube.

SUMMARY.

1. Distillation of ergosterol and recrystallisation of ergosteryl ethyl carbonate are unsatisfactory methods for purifying ergosterol, and recrystallisation from alcohol-benzene (2:1) does not invariably yield a pure product.

2. A high degree of purity is attained by benzylation of ergosterol, recrystallisation of the benzoate from ethyl acetate at 37°, and hydrolysis.

3. Ergosteryl benzoate is trimorphic.

4. No variation has been found in the properties of purified yeast ergosterol from different sources, and no evidence of the existence of natural isomerides has been obtained.

5. When ergosterol is kept over a dehydrating agent in air it takes up five

atoms of oxygen. This oxidation is rapid in the case of distilled material, but also takes place with recrystallised hydrated material after an induction period.

I wish to express my thanks to Dr O. Rosenheim, who initiated this work, for his constant advice and encouragement, and also to Mr T. A. Webster, Miss H. M. Bruce and Miss C. Fischmann, who carried out the biological tests, and to Mr R. G. C. Jenkins, who carried out the spectrography.

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REFERENCES.

- Askew, Bourdillon, Bruce, Jenkins and Webster (1930). *Proc. Roy. Soc. Lond. B* **107**, 76.
Bills and Cox (1929). *J. Biol. Chem.* **84**, 455.
—— and Honeywell (1928). *J. Biol. Chem.* **80**, 15.
—— and McDonald (1930). *J. Biol. Chem.* **88**, 337.
Callow (1930). *Chem. Ind.* **49**, 991.
—— (1931). *Biochem. J.* **25**, 87.
Heilbron, Sexton and Spring (1929). *J. Chem. Soc.* 926.
Reindel and Detzel (1929). *Liebig's Ann.* **475**, 48.
Tanret (1908). *Ann. Chim. Phys.* (8), **15**, 314.
Wieland and Asano (1929). *Liebig's Ann.* **473**, 300.
Windaus and Grosskopf (1922). *Z. physiol. Chem.* **124**, 8.
—— and Rygh (1928). *Nachr. Ges. Wiss. Göttingen, Math.-Phys. Kl.* 202.

XIII. OCCURRENCE OF α -DIHYDROERGOSTEROL AS AN IMPURITY IN YEAST ERGOSTEROL.

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THE fractionation of the benzoates obtained from ordinary ergosterol [Callow, 1930, 1931] has led to the isolation of α -dihydroergosterol, the occurrence of which among the yeast sterols has not hitherto been reported. This compound is the only one found present in sufficient amount for separation and identification.

The attempt to isolate α -dihydroergosteryl benzoate by means of fractional crystallisation was a laborious process. The more soluble fractions of the benzoates from ordinary ergosterol crystallised from ethyl acetate in stable leaflets isomorphous with the unstable form of ergosteryl benzoate, and the properties of the material closely resembled those of neosteryl benzoate [Wiand and Asano, 1929]. On repeated recrystallisation from other solvents, however, the laevorotation diminished and the melting point rose, whilst the product still contained a large proportion of ergosteryl benzoate. It became evident that the unstable leaflet form of ergosteryl benzoate formed a stable mixed crystal phase with another benzoate crystallising in isomorphous leaflets. After the accumulation of larger quantities of material an attempt was made to remove the ergosterol by fractional crystallisation from ethylene dichloride, which was the best of the various solvents tried, but the results indicated that the isolation of any considerable quantity by this method would be impracticably slow. Recourse was had therefore to a chemical method of separation. It was found that partial bromination decomposed the ergosteryl benzoate preferentially, and ultimately the ergosterol content of the product of hydrolysis was reduced to 0.25 %. The last traces of ergosterol could be removed by ultra-violet irradiation.

The identity of the sterol thus isolated with α -dihydroergosterol, indicated by the correspondence of the properties with those recorded in the literature, was confirmed by comparison with an authentic specimen. The *p*-nitrobenzoate, which was prepared for the first time, is remarkable for the large range of temperature (46°) over which the anisotropic liquid phase is stable. This range is small in all derivatives of ergosterol hitherto examined, and the crystalline liquid is difficult to observe at all in some cases [cf. Gaubert, 1908].

No indication of the presence of sterols other than α -dihydroergosterol was obtained, although their presence in small amount in the most soluble

fractions is not impossible. The similarity of the neosteryl benzoate of Wieland and Asano [1929] to the products obtained in the initial stages of the separation of α -dihydroergosteryl benzoate strongly suggests that the former material was a similar isomorphous mixture. The data given, in particular the absorption spectrum, and the comments made by Wieland and Gough [1930] give some support to this suggestion. It was actually found that a mixture of ergosterol and α -dihydroergosterol yielded a benzoate, resembling neosteryl benzoate, which formed homogeneous crystals from ethyl acetate and showed little change in specific rotation after recrystallisation from that solvent. The existence of neosterol as a chemical entity cannot of course be denied without examining a sterol mixture similar to that used by Wieland and Asano as their starting product. The "cerevisterol" of Bills and Honeywell [1928], which would be expected to yield a benzoate of high melting point and slight solubility, was not present, nor has any indication of its presence been obtained in numerous other specimens of ergosterol in this laboratory.

It is evident that in the specimens of ergosterol examined the variations in specific rotation could be accounted for almost entirely by the presence of α -dihydroergosterol in varying amounts, and that this is the only considerable impurity. The specific rotation of a mixture of 95 % of ergosterol ($+ \text{H}_2\text{O}$; $[\alpha]_{5461}^{20} - 167.2^\circ$) with 5 % of α -dihydroergosterol ($+ 0.5 \text{ C}_2\text{H}_5\text{OH}$; $[\alpha]_{5461}^{20} - 21.1^\circ$) would be $[\alpha]_{5461}^{20} - 159.9^\circ$, which is close to the average values shown by ordinary "pure" ergosterol.

α -Dihydroergosterol shows absorption only in the extreme ultra-violet, and it is unaffected by radiation of wave-lengths above $275 \mu\mu$. Windaus, Gaede, Köser and Stein [1930], in a recent paper describing the products of over-irradiation of ergosterol through "Uviolglas," record that 4 % of the product was precipitated as a digitonide which proved to be that of α -dihydroergosterol. In the light of the results now described it is probable that the latter was present in their starting material.

The recognition of α -dihydroergosterol as a companion of ergosterol in yeast indicates that the simultaneous occurrence of sterols in different stages of saturation is a general phenomenon connected with the synthetic cellular activity of plants and animals. Evidence for such a view has been accumulating during the course of this work. The occurrence of dihydroergosterol along with fungisterol and ergosterol in ergot [Heyl and Swoap, 1930] is analogous to its association with zymosterol and ergosterol in yeast. Dihydrositosterol has been found to be a constant companion of sitosterol in cereals [Anderson, Nabenhauer, and Shriner, 1927] and in the soya bean [Bonstedt, 1928]. The latter also contains a doubly unsaturated sterol, stigmasterol, which is found associated with sitosterol in the Calabar bean and cacao. Finally, it has recently been found [Schönheimer, Behring, Hummel, and Schindel, 1930], that the main sterol constituent of animal cells, cholesterol, contains dihydrocholesterol as a constant admixture. The significance of the occurrence of these saturated sterols is uncertain. The last named authors consider that dihydro-

cholesterol is formed by hydrogenation of cholesterol, but there is as yet little evidence to indicate whether the formation of the more saturated sterols by reduction is general. It is possible that in some cases they are intermediates in the formation of the unsaturated compounds.

EXPERIMENTAL.

Material. The specimens of ergosterol which were the starting material for this work were the same as those used in the purification of ergosterol previously described [Callow, 1931]. After benzylation of the crude ergosterol with benzoyl chloride in pyridine and separation of the material which crystallised out directly, a final fraction (10–15 % of the total yield) was obtained by the addition of water to the pyridine solution. The presence of the benzoate of another sterol was immediately evident when this fraction was recrystallised from ethyl acetate, for a new stable crystalline form appeared—homogeneous leaflets belonging to the monoclinic system in the case of the least pure specimen of ergosterol (F; $[\alpha]_{5461}^{20} - 137.2^\circ$), and mixtures of leaflets with needles of ergosteryl benzoate in the case of other specimens ($[\alpha]_{5461}^{20} - 156.9$ to -161.2°). In addition, the higher fractions of the benzoates from specimen F yielded mixtures of needles and leaflets, and a small quantity of leaflets was obtained from the mother-liquors of the higher fractions of the benzoates from other specimens. Mixtures of leaflets and needles could be separated by dissolving in boiling ethyl acetate and collecting fractions at 37° and -4° . In this way batches of leaflets were collected with m.p. ranging from 159 – 165° to 165 – 170° , and values of $[\alpha]_{5461}^{20}$ ranging from -59° to -65° . In a typical case 50 g. of specimen A of ergosterol gave a total of 6.8 g. of leaflets.

Some difficulty was encountered in the initial stages of the work owing to the change which took place in ethyl acetate mother-liquors kept exposed to the air. This change, the nature of which is still uncertain, was indicated by an increase in solubility, yellowing of the solution on concentration, and the subsequent deposition of poorly crystallised products. The lower crystalline fractions from ethyl acetate also showed signs of oxidation when stored in contact with air. Simultaneously, the material developed an intense colour reaction with a 25 % solution of mercuric acetate in nitric acid ("mercury reagent") [Rosenheim and Callow, 1931]. When a chloroform solution of the material was mixed with the reagent and shaken the chloroform layer turned bluish-red, changing to blue, and then, after an interval, to green, the acid layer remaining colourless. In the ultimate systematic fractionation the composition of all fractions obtained was checked by the use of this reagent.

After hydrolysis with 3 % alcoholic potassium hydroxide these fractions of altered material yielded a brown solution from which a colourless sterol mixture separated, and the latter, when re-benzoylated, gave a mixture of benzoates which showed only the usual ergosterol reaction with the "mercury reagent" (chloroform layer pink \rightarrow orange \rightarrow yellow). The difficulty of working up sterol mixtures from the mother-liquors of technical ergosterol may be due

to the presence of similar transformation products of ergosterol, and the re-saponification process which Wieland and Gough [1930] applied to such material may owe its usefulness to the destruction of these products by alkali.

Separation of mixtures rich in α -dihydroergosteryl benzoate by fractional crystallisation. After preliminary investigations which indicated that the fractions of the benzoate crystallising in leaflets contained a large proportion of ergosteryl benzoate, and after trials with various solvents, a systematic fractionation was begun with 180 g. of material by crystallisation from ethylene dichloride. The benzoate was dissolved in eight parts of boiling ethylene dichloride in an atmosphere of nitrogen, cooled, and fractions were collected at 37° and at -4° . The mother-liquors were either used for the recrystallisation of lower fractions or were evaporated in a current of nitrogen. In this way at one end of the system a series of less soluble fractions was obtained with decreasing laevorotation and rising m.p., whilst ergosteryl benzoate became concentrated in the more soluble fractions and ultimately separated in needles. The progress of the separation is illustrated by the following figures for successive head fractions: (1) m.p. $176-181^\circ$, $[\alpha]_{5461}^{20} - 55.8^\circ$; (2) m.p. $179-183.5^\circ$, $[\alpha]_{5461}^{20} - 45.5^\circ$; (3) m.p. $180.5-187^\circ$, $[\alpha]_{5461}^{20} - 38.7^\circ$; (4) m.p. $185-190^\circ$, $[\alpha]_{5461}^{20} - 32.2^\circ$, 12.1 g.; (5) m.p. $187.5-192^\circ$, $[\alpha]_{5461}^{20} - 26.6^\circ$, 6.3 g. When the m.p. and specific rotation were plotted on curves against number of recrystallisations it appeared that at least six further recrystallisations would be necessary before practical constancy could be attained.

Removal of ergosterol by bromination. Bromination of the mixed benzoates was initially tried in the hope that the sterol, then unidentified, accompanying ergosterol would form an easily separable bromide. Preliminary experiments, however, in which bromination was carried out in ether, chloroform or carbon tetrachloride, yielded no product sparingly soluble in alcohol when excess of bromine was used. When smaller proportions of bromine, preferably in carbon tetrachloride solution, were used, the addition of alcohol precipitated products free from bromine, and these were found to have the same properties as the products obtainable from the same starting material by fractional crystallisation.

The following method was found suitable for removing ergosterol. The mixture of benzoates (30.7 g.; containing about 30 % of ergosterol) was dissolved in carbon tetrachloride (400 cc.), and a solution of bromine in carbon tetrachloride (31 cc. of a 5 % solution by volume; about 2.5 mols./mol. of ergosterol) was added slowly with shaking. After allowing to stand for a few minutes, 95 % alcohol (1500 cc.) was added. The precipitate (18.8 g.) was collected, washed with alcohol, and recrystallised from ethylene dichloride. The slightly discoloured product had m.p. $189-193^\circ$, $[\alpha]_{5461}^{20} - 12^\circ$, and contained 3 % of ergosterol, determined colorimetrically. The ergosterol content was not reduced below 1 % by repeating the bromination. A specimen obtained in this way contained 1 % of ergosterol, and had m.p. $194-196^\circ$, $[\alpha]_{5461}^{20} - 13.0^\circ$. (Found (micro): C, 83.4; H, 10.0 %; $C_{34}H_{48}O_2$ requires C, 83.5;

H, 9.9 %.) It was found best, when dealing with fractions of high ergosterol content, to remove the ergosterol by two successive partial brominations.

α -Dihydroergosterol. Hydrolysis of the benzoate (14 g.; 3 % ergosteryl benzoate) was carried out by boiling for 45 minutes with 3 % alcoholic potassium hydroxide solution (800 cc.). Water was added to the hot solution until it was slightly cloudy, and the crystals which separated on cooling were collected, washed, dried, and recrystallised from alcohol-benzene (2:1). α -Dihydroergosterol (8.6 g.), containing 0.25 % of ergosterol, separated in leaflets, m.p. 172–174°, containing 0.5 mol. of alcohol of crystallisation. The solvent-free substance had $[\alpha]_{D}^{20} - 23.8^\circ$. (Found: loss at 130°/10 mm. over P_2O_5 , 5.7, 5.7 %, and, in solvent-free substance (micro), C, 83.6; H, 11.6 %. $C_{27}H_{44}O$, 0.5 C_2H_5OH requires loss, 5.65 %. $C_{27}H_{44}O$ requires C, 84.3; H, 11.5%.) The digitonide was precipitated by the addition of 1 vol. of a 1 % solution of digitonin in 90 % alcohol to 2 vols. of a 0.1 % solution of the sterol in 90 % alcohol. (Found: digitonide: sterol = 3.9:1.)

A portion of this specimen of α -dihydroergosterol was irradiated in 0.5 % solution in benzene by a quartz mercury vapour lamp for a period sufficient to destroy the ergosterol present. After evaporation and crystallisation of the residue twice from 95 % alcohol (charcoal), the product, freed from solvent at 130°/10 mm. (loss 5.9 %), had m.p. 172.5–174° (unchanged by mixture with an authentic specimen of α -dihydroergosterol of the same m.p.), and $[\alpha]_{D}^{20} - 23.0^\circ$. (Windaus and Brunken [1928] give m.p. 173–174°, $[\alpha]_{D}^{17} - 20.4^\circ$; Heilbron and Sexton [1929] give m.p. 173°, $[\alpha]_{D}^{20} - 21.7^\circ$.) The colour reactions were as follows: Liebermann-Burchard: momentary purple \rightarrow dark-blue \rightarrow bottle-green \rightarrow emerald. Bromine (Tortelli-Jaffé): negative in dilute solution; in concentrated solution, yellow \rightarrow emerald \rightarrow blue-green. Iodine [Rosenheim and Callow, 1931]: negative. Salkowski: chloroform colourless, acid deep yellow with green fluorescence. Antimony trichloride: negative, red after standing. Trichloroacetic acid: negative, dull green with light green fluorescence after 6 hours. Mercuric acetate in nitric acid [Rosenheim and Callow, 1931]: negative. The slow, secondary reactions with antimony trichloride and trichloroacetic acid, which have not been described before, were also given by an authentic specimen of α -dihydroergosterol. The digitonide was precipitated in 90 % alcohol. (Found: digitonide: sterol = 4:1.)

Spectrographic examination of purified α -dihydroergosterol in 0.1 % alcoholic solution showed that absorption was negligibly small at wave-lengths exceeding 250 $\mu\mu$, but then increased rapidly down to 230 $\mu\mu$, the limit of measurement [cf. De Boe, 1930]. Investigation of the effect of irradiation in alcoholic solution by a quartz mercury vapour lamp with and without filters [cf. Askew *et al.*, 1930] indicated that a small amount was destroyed by the total radiation or by wave-lengths between 210 $\mu\mu$ and 280 $\mu\mu$, and a slight diminution of absorption occurred. The product of irradiation by the unscreened lamp, in which about 3 % was destroyed, showed no antirachitic activity in rats in doses of 8 γ daily. Irradiation with light of wave-lengths

exceeding $275\mu\mu$, under conditions in which ergosterol is destroyed to the extent of 50 %, caused no significant alteration of the absorption or of the amount precipitated by digitonin. A trace of a substance of high melting point appeared to be formed.

Derivatives of α -dihydroergosterol. These were prepared from material containing 0.25 % of ergosterol.

The *benzoate*, prepared with excess of benzoyl chloride in pyridine, and recrystallised, had M.P. $192-195^\circ$, $[\alpha]_{5461}^{20} - 13.6^\circ$. The M.P. of the benzoate prepared from 10 mg. of an authentic specimen of α -dihydroergosterol was $188-194^\circ$, and a mixture melted at $190-195^\circ$. Heyl and Swoap [1930] record M.P. $153-155^\circ$, but from their description it is evident that, owing to the use of insufficient benzoyl chloride, their material must have been a mixture of free sterol and benzoate.

The *acetate*, prepared by boiling with acetic anhydride for 20 minutes, and recrystallised from ethyl acetate, had M.P. $178-180^\circ$, $[\alpha]_{5461}^{20} - 24.2^\circ$. (Windaus and Brunken [1928] give M.P. $180-181^\circ$. Heilbron, Johnstone, and Spring [1929] give M.P. $179-180^\circ$, $[\alpha]_{5461}^{20} - 25.3^\circ$.) (Found (micro): C, 81.9; H, 10.9 %. $C_{29}H_{46}O_3$ requires C, 81.6; H, 10.9 %.)

The *p-nitrobenzoate* was prepared by adding a solution of *p*-nitrobenzoyl chloride (0.3 g.) in chloroform (2 cc.) to α -dihydroergosterol (9.1 g.) in pyridine (2 cc.). The reaction mixture was poured into water, chloroform was removed by evaporation, and the solid was collected, washed with hot alcohol, and recrystallised twice from 80 % aqueous pyridine. It separated in lanceolate plates, melting at 202° to a cloudy, anisotropic liquid, clearing point 248° . It separated from other solvents in gelatinous form. (Found (micro): C, 76.9; H, 8.8 %. $C_{34}H_{47}O_4N$ requires C, 76.5; H, 8.9 %.)

The number of ethylenic linkings in the acetate and benzoate was determined by titration with bromine in carbon tetrachloride according to the modification of Kaufmann's method described by Reindel and Niederländer [1929]. (Found: acetate, 1.9, 1.8; benzoate, 1.7. Control analyses: ergosteryl benzoate, 2.4, 2.4; cholesteryl acetate, 1.1.)

Mixtures resembling neosterol. Neosterol, M.P. $164-165^\circ$, $[\alpha]_D^{24} - 105^\circ$, was isolated by Wieland and Asano [1929] from material derived from the mother-liquors of yeast ergosterol, and the benzoate was described as leaflets having M.P. $173-175^\circ$, $[\alpha]_D^{24} - 50.6^\circ$. The sterol gave the same colour reactions as ergosterol, and its absorption in the ultra-violet [Wieland and Gough (with Page), 1930] was almost identical. The fractions of benzoate crystallising in leaflets encountered in the first stages of the separation strongly resembled the description of neosteryl benzoate. One fraction, for example, had M.P. $170-177^\circ$, $[\alpha]_{5461}^{20} - 60.0^\circ$, and another had M.P. $172-176^\circ$, $[\alpha]_{5461}^{20} - 63.8^\circ$. However, recrystallisation of the first from ethyl acetate and twice from acetone gave a product of M.P. $173-185^\circ$, $[\alpha]_{5461}^{20} - 47.4^\circ$, and two recrystallisations of the second from ethylene dichloride at 37° gave a product of M.P. $180.5-185^\circ$, $[\alpha]_{5461}^{20} - 46.7^\circ$. The hydrolysis of these products yielded sterols which, re-

crystallised from alcohol, had, respectively, m.p. 165.5–170°, $[\alpha]_{5461}^{20} - 86.9^\circ$ and m.p. 168–172°, $[\alpha]_{5461}^{20} - 91.5^\circ$. (Found (micro), in material dried at 130°/10 mm. over P_2O_5 : C, 83.3; H, 9.8 %. $C_{34}H_{48}O_2$ requires C, 83.6; H, 9.8 %.) The antirachitic potency after irradiation was in each case half that of ergosterol, and the extinction coefficients of the alcoholic solution at 281.7, 271.5, and 247.3 $\mu\mu$ were 55–57 % of those of ergosterol. In addition, colorimetric measurement of the second specimen (described below) indicated the presence of about 55 % of ergosterol.

It was thus evident that the material resembling neosteryl benzoate was, in fact, an isomorphous mixture, and this was confirmed, after the recognition of α -dihydroergosterol as one of the constituents, by the preparation of a homogeneous mixed benzoate of ergosterol and α -dihydroergosterol. Ergosterol (0.7 g.) and α -dihydroergosterol (0.3 g.) were dissolved in pyridine (10 cc.) and treated with benzoyl chloride (2.5 cc.). The crude product, separated by pouring into water and washing with alcohol, had m.p. 167–174°, $[\alpha]_{5461}^{20} - 65.4^\circ$. It crystallised from ethyl acetate in leaflets, m.p. 170–175°, $[\alpha]_{5461}^{20} - 62.5^\circ$. This entirely confirms the conclusions as to the composition of the early fractions of benzoates, and lends additional support to the suggestion that neosteryl benzoate is also such a mixture.

Colorimetric determination of ergosterol. The blue colour obtained when a chloroform solution of ergosterol or ergosteryl benzoate is mixed with a 90 % aqueous solution of trichloroacetic acid [Rosenheim, 1929] attains a maximum intensity after 5 minutes, and is then readily matched by a combination of blue and yellow Lovibond tints in the Rosenheim-Schuster colorimeter [Rosenheim and Schuster, 1927]. The intensity of the blue is maximal when the reagent and the chloroform solution are mixed in the proportion 2:1, and is then approximately proportional to the concentration of ergosterol, thus providing a rapid method for the determination of ergosterol or the benzoate. Example: a 0.2 % solution of a sterol mixture gave 8.1 blue units (+ 2.7 yellow) in a 10 mm. cell, whilst a 0.1 % solution of ergosterol gave 7.5 blue units (+ 3.2 yellow); the mixture therefore contained $0.1/0.2 \times 8.1/7.5 \times 100 = 55$ % of ergosterol, a result which was in agreement with that of spectroscopic analysis.

SUMMARY.

1. Ordinary yeast ergosterol commonly contains considerable amounts of α -dihydroergosterol.
2. α -Dihydroergosteryl benzoate has been isolated by fractionation of the benzoates from yeast ergosterol, followed by a process of partial bromination which destroys the ergosterol preferentially. α -Dihydroergosterol has been identified and characterised by the preparation of derivatives.
3. The neosterol of Wieland and Asano [1929] has not been found, but the benzoates of ergosterol and α -dihydroergosterol, as well as the free sterols, form mixed crystals whose properties agree with those of neosteryl benzoate and neosterol, respectively.

4. The coloration given by ergosterol with trichloroacetic acid has been applied to its determination.

I wish to express my gratitude to Dr O. Rosenheim for his constant advice and criticism, and to thank Mr T. A. Webster, Miss H. M. Bruce, and Miss C. Fischmann for carrying out the biological tests, and Messrs T. C. Angus, F. A. Askew, and J. St L. Philpot for assistance in the irradiation and spectrographic work.

REFERENCES.

- Anderson, Nabenhauer and Shriner (1927). *J. Biol. Chem.* **71**, 389.
Askew, Bourdillon, Bruce, Jenkins and Webster (1930). *Proc. Roy. Soc. Lond. B* **107**, 91.
Bills and Honeywell (1928). *J. Biol. Chem.* **80**, 15.
Bonstedt (1928). *Z. physiol. Chem.* **176**, 269.
Callow (1930). *Chem. Ind.* **49**; *Proc. Biochem. Soc.* 991.
—— (1931). *Biochem. J.* **25**, 79.
De Boe (1930). *Bull. Acad. Roy. Méd. Belgique*, [v], **10**, 336.
Gaubert (1908). *Compt. Rend. Acad. Sci.* **147**, 498.
Heilbron, Johnstone and Spring (1929). *J. Chem. Soc.* 2248.
—— and Sexton (1929). *J. Chem. Soc.* 921.
Heyl and Swoap (1930). *J. Amer. Chem. Soc.* **52**, 3688.
Reindel and Niederländer (1929). *Liebig's Ann.* **475**, 147.
Rosenheim (1929). *Biochem. J.* **23**, 47.
—— and Callow (1931). *Biochem. J.* **25**, 74.
—— and Schuster (1927). *Biochem. J.* **21**, 1329.
Schonheimer, Behring, Hummel and Schindel (1930). *Z. physiol. Chem.* **192**, 73.
Wieland and Asano (1929). *Liebig's Ann.* **473**, 300.
—— and Gough (1930). *Liebig's Ann.* **482**, 36.
Windaus and Brunken (1928). *Liebig's Ann.* **460**, 225.
—— Gaede, Köser and Stein (1930). *Liebig's Ann.* **483**, 17.

XIV. SOME PECULIARITIES IN THE GLYCERIDE STRUCTURE OF LAUREL FATS. ✓

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THE broad differences between the types of synthesis that occur in three important sources of natural fats, the seed kernel, the fruit pulp and the animal tissues, were discussed in a previous communication [Collin and Hilditch, 1929], where it was suggested that, whilst the syntheses in the fruit pulp and animal tissues were very similar as far as the manner in which the available fatty acids were arranged as glycerides, the kernel synthesis evidently proceeded in a rather different way, and kernel fats were thereby endowed with a characteristic structure. It was inferred from a consideration of the association ratios (the measure of the degree of dispersion of the unsaturated acid throughout the fat) of the various kernel fats studied, that their structure depends essentially upon an unusually thorough dispersion of the fatty acids among the completed glyceride molecules. Evidence of such a structure was given by all the kernel fats examined with the exception of a sample of the commercially expressed oil of laurel (*Laurus nobilis*). Although this sample was expected to contain a certain amount of pulp fat and therefore to show some irregularity, the structure it actually disclosed was so anomalous that specimens of the true laurel kernel and laurel pulp fat from *Laurus nobilis* berries were prepared in these laboratories and analysed by similar methods for further and more reliable information.

The suggestion in the earlier paper [1929, p. 1278], that the observed abnormality might be due to admixed pulp fat, must be withdrawn for it now appears that the pulp fat is a perfectly normal specimen of its class, and that the anomaly lies entirely in the kernel fat, where the expected even mixed glyceride structure is absent and is replaced by one of extreme heterogeneity.

According to the association ratios, which are approximately constant throughout the kernel fat group, the persistent saturated-unsaturated glyceride mixture in a normal kernel fat contains about 1.4 molecules of saturated acid associated with every molecule of unsaturated acid; and, apparently, when the normal synthesis is taking place, no fully saturated glycerides are formed until this ratio is exceeded in the original mixture of acids. When the proportion of saturated acids in the mixture exceeds this ratio, formation of

saturated-unsaturated glycerides with an average association ratio of 1.4:1 continues until all the unsaturated acids are accounted for and the surplus saturated acids appear as fully saturated glycerides.

The mixture of fatty acids in the laurel kernel fat now studied had the following composition (excluding unsaponifiable): lauric 43.1 %, palmitic 6.2 %, oleic 32.3 %, linoleic 18.4 %. The molecular ratio of saturated to unsaturated acid in this mixture is very near the critical value of 1.4:1 and hence is ideally adjusted for complete conversion into mixed saturated-unsaturated glycerides with formation of no, or at the most of insignificant amounts of, fully saturated material. Actually, however, the kernel fat contains about 36 % of saturated glycerides, and here obviously the more usual mechanism of kernel fat synthesis has not been in operation. The composition of the fatty acid mixture from the saturated glycerides (lauric 95.5 %, palmitic 4.5 %) shows, moreover, that trilaurin is the predominant constituent to the extent of about 90 % of the whole; and it may be recalled that Bömer and Ebach [1928] were also able to isolate about 30 % of trilaurin from laurel oil by direct crystallisation. It should also be emphasised, in view of what follows, that although the saturated portion of the fat is so rich in lauric glycerides the remainder contains only about a quarter of the total lauric acid present in the fat.

A further difficulty is provided by the behaviour of the unsaturated acids. The limiting values for the content of tri-unsaturated glycerides can be calculated from the association ratio of the mixed saturated-unsaturated (and tri-unsaturated) glycerides and must lie between 6 and 31 %. If the heterogeneity observed in the saturated acids extended to the unsaturated acids, as was expected, then the true value would approach the higher limit. In attempting to trace such a correspondence, a part of the fat was hydrogenated as completely as possible (thus transforming all the tri-unsaturated constituents to tristearin) and fractionally crystallised from ether. Although such a method is not strictly quantitative, tristearin is so insoluble in cold ether that it may be isolated fairly completely; in this case the amount of tristearin found was small enough to justify the conclusion that the true value for the tri-unsaturated content approaches the minimum. Thus the unsaturated acids show no tendency to link up with each other after the example of the saturated acids, but distribute themselves in the characteristic way among the residual palmitic and lauric acids left over from the abnormal saturated glyceride formation.

It is difficult to reconcile the behaviour of the two groups of acids without concluding either that they were in different parts of the seed at the time of their conversion into glycerides, or that they arrived in the seed over different periods. The latter suggestion not only covers the behaviour of the acids but also offers some explanation of the peculiar glyceride structure of the fat; for, assuming that the bulk of the lauric acid appears alone in the kernel during the early stages of ripening, then trilaurin alone would be synthesised until,

as the ripening proceeded, the appearance of the unsaturated acids and the rest of the lauric acid would allow the kernel to resume the normal mixed glyceride synthesis, without however affecting the trilaurin already formed. Palmitic, the other saturated acid, would appear from the experimental data to have spread itself over the whole process, reaching its greatest concentration in the second stage of the synthesis along with the unsaturated acids.

A stepwise construction of the fat on these lines would therefore readily account for its structural divergence from the ordinary kernel fat.

EXPERIMENTAL.

The outer pulp was removed without difficulty from the laurel berries leaving a cleanly separated kernel; both pulp and kernels were steeped in acetone to dry and to remove excess fat, ground to a meal, extracted with ether in a Soxhlet and the fat combined with the acetone extract. The berries yielded 70 % kernel (fat content 14 %) and 30 % pulp (fat content 37 %); or, calculated on the original weight of berries, 10.1 % kernel fat and 11.2 % pulp fat.

The kernel fat was solid and light brown in colour. The pulp fat was a dark green liquid, which on long standing deposited small nodules of solid. They possessed the following characteristics:

	Sap. equiv.	Iodine value
Kernel fat	257.4	84.0
Pulp fat	285.7	113.1

The preliminary treatment of each fat followed the same course. They were saponified with 10 % alcoholic potash for 6 hours and the liberated acids separated into "liquid" and "solid" acids by a modification of the Twitchell process. In each case, also, before esterification the "solid" and "liquid" acids were reconverted to soap by the addition of excess of alcoholic potash and the unsaponifiable material removed by repeated extraction with ether.

	"Solid" acids g.	Non-sap. g.	"Liquid" acids g.	Non-sap. g.
Kernel fat	32.5	—	39.3	20.2
Pulp fat	26.0	0.6	55.1	7.7

The methyl esters of the purified "solid" and "liquid" acids were prepared and fractionally distilled in the usual manner, and the analytical data thus obtained are summarised below.

<i>Laurel kernel fat.</i>							% of fatty acids	
							Total	Excluding non-fatty material
							“Solid” acids (35·3 %)	“Liquid” acids (64·7 %)
Lauric	25·0	8·2
Palmitic	4·8	—
Oleic	5·5	19·4
Linoleic	—	14·2
Unsaponifiable	—	0·9
„	(extracted before methylation)						—	22·0
							33·2	43·1
							4·8	6·2
							24·9	32·3
							14·2	18·4
							22·9	—

Laurel pulp fat.

						% of fatty acids		
						"Solid" acids (29.8 %)	"Liquid" acids (70.2 %)	Excluding non-fatty material
Lauric	1.3	1.1	2.4
Palmitic	18.3	—	18.3
Oleic	8.0	48.9	56.9
Linoleic	1.5	11.1	12.6
Unaponifiable	—	0.5	—
,, (extracted before methylation)						0.7	8.6	—

Molecular ratio of saturated to unsaturated acids 0.34:1.

Estimation of the fully saturated glycerides.

This was carried out in the usual way by treating a solution of the fat in acetone with powdered potassium permanganate and isolating the unchanged saturated glycerides from the oxidation mixture by washing with ammonia and potassium carbonate.

Laurel kernel fat.

150 g. of the kernel fat gave 42.0 g. of crude fully saturated glycerides (iodine value 1–2), and these, on further purification by boiling with aqueous potassium carbonate yielded:

- (a) 31.9 g. (corr. wt.) neutral fat, sap. equiv. 211.1 (acid value 0.4);
- (b) 3.2 g. (corr. wt.) fat extracted by ether, sap. equiv. 231.2 (acid value 6.2);
- (c) 6.9 g. (corr. wt.) mainly acidic material, sap. equiv. 199.2 (acid value 45.7).

Assuming that the acidic matter present in (b) and (c) is azelaodilaurin (acid value 89.5), the proportion of fully saturated glycerides in the original material is 26.6 %; allowing for the 22 % of non-fatty compounds present in the original, the proportion of fully saturated glycerides in the true kernel fat is therefore 34.2 %.

The fully saturated portion (a) was converted into methyl esters, which gave the following results on fractionation:

g.	B.P./1 mm.	Sap. equiv.
4.28	73–93°	210.2
5.66	93	213.2
7.62	93	213.4
3.36	Residue	251.4*

* Residual esters, freed from unaponifiable matter, sap. equiv. 228.0.

This corresponds with a composition of lauric acid 94.1 %, palmitic acid 4.3 % and unaponifiable matter 1.6 %, or, excluding unaponifiable matter, lauric acid 95.5 % and palmitic acid 4.5 %.

The general composition of 100 parts of the original kernel fat, based on the preceding data, is as follows:

	Total fat	Fully saturated glycerides	Mixed saturated-unsaturated or tri-unsaturated glycerides (by difference)	
	100 g.	26.6 g.	73.4 g.	
Unaponifiable	22.0	0.4	21.6	
Glyceryl residue	3.9	1.5	2.4	
				Molecular ratios
Lauric	31.9	23.6	8.3	415
Palmitic	4.6	1.1	3.5	135
Oleic	23.9	—	23.9	848
Linoleic	13.7	—	13.7	489

The preponderance of lauric acid in the fully saturated portion and of palmitic acid in the rest of the fat may be noted. From the figures in the final column it appears that the "association ratio" in the mixed glycerides is about 0.4 mol. saturated acids per mol. of unsaturated acids. Consequently, of 100 parts by weight of the original kernel fat (containing 22 % non-fatty matter), 26.6 parts consist of fully saturated glycerides as described, whilst about 6.31 parts are tri-unsaturated glycerides, according as the amount of mono-unsaturated-disaturated glycerides lies between nil and 20, and that of di-unsaturated-monosaturated glycerides between 45 and nil.

Tristearin content of the hydrogenated fat. The original kernel fat (40 g.) was hydrogenated repeatedly with 5 g. nickel catalyst until the iodine value fell to 14. Great difficulty was experienced in attaining even this degree of saturation, probably because the large amount of non-fatty matter was either toxic to the catalyst or was itself difficult to hydrogenate; the residual iodine value is almost certainly to be attributed to the non-fatty constituents. The tri-unsaturated glycerides being thus converted to tristearin, the mixture (39.4 g.) was fractionally crystallised from ether. From the first crystallisation of the hydrogenated fat the following fractions were obtained by successive concentrations of the filtrate from each crop of crystals:

Crop	g.	M.P.
A	5.7	64–65
B	4.8	49–50°
C	6.1	41–43°
D	9.4	33.5–35°
E	13.4	— (ex final mother-liquors)

On recrystallisation of crop A, a first fraction (2.8 g., M.P. 66.5–67.5°, sap. equiv. 291.2) was obtained, whilst the more soluble part melted at about 64–65° and had a sap. equiv. of 284.0. Recrystallisation of crop B yielded only a minute amount of material melting at 64–65°, the residue having a maximum melting point of about 52–53°. Since even the least-soluble and highest-melting fraction from crop A is not entirely tristearin, it is quite clear from this examination that the proportion of tri-unsaturated glycerides in the original fat cannot greatly have exceeded the minimum amount shown to be present by the previous quantitative oxidation experiments.

Laurel pulp fat.

100.5 g. of the pulp fat yielded 2.7 g. of saturated material (sap. equiv. 267.5). This corresponds with a fully saturated glyceride content of approximately 3 % on the glycerides present in the pulp fat.

The saturated compounds gave on hydrolysis fatty acids which, after removal of unsaponifiable matter, possessed an equivalent of 255.6 and a melting-point of 60–62° (unaltered on admixture with pure palmitic acid); the glyceride present was thus substantially tripalmitin.

The proportion of fully saturated glycerides, although small, is thus quite well defined, and it is interesting to observe that it is coincident with that of the corresponding synthetic triglyceride mixture as indicated by the graph correlating the proportion of fully saturated glycerides with the proportions of saturated and unsaturated acids in the mixed fatty acids of a synthetic triglyceride [Bhattacharya and Hilditch, 1930]. In common with other fruit pulp fats and with animal tissue fats, therefore, the glyceride structure of laurel pulp fat is closely similar to that of the corresponding synthetic mixed triglycerides.

In conclusion the author wishes to thank Professor T. P. Hilditch for his advice and encouragement during the investigation.

REFERENCES.

- Bhattacharya and Hilditch (1930). *Proc. Roy. Soc. Lond. A* **129**, 468.
Bömer and Ebach (1928). *Z. Unters. Nahr. Genussm.* **55**, 501.
Collin and Hilditch (1929). *Biochem. J.* **23**, 1273.

XV. IODIMETRIC DETERMINATION OF REDUCING SUGARS IN THE APPLE.

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It was pointed out by Judd [1920] that the polarimetric method in combination with the copper reduction method for the determination of fructose and glucose in mixtures of these sugars was unsuitable for the analysis of apple extracts, not only because of the low optical rotation of the extracts, but because they are always coloured and frequently viscous. Evans [1928] investigated various methods of clearing the extracts and found that considerable dilution was necessary for satisfactory clarification. In order to obtain a sufficiently large reading on the polarimeter the cleared solutions had to be evaporated under reduced pressure to a suitable concentration, making the analysis very tedious. Both these workers recommended the use of the iodimetric method [Baker and Hulton, 1920; Cajori, 1922; Willstätter and Schüdel, 1918] in combination with copper reduction for the estimation of fructose and glucose in the apple. This method is based on the oxidation of the sugars by alkaline iodine, and some disagreement existed as to the extent of the oxidation until Hinton and Macara [1924] reinvestigated the reaction. They established the fact that glucose was quantitatively oxidised to gluconic acid in 10 minutes at room temperature, provided not more than half the iodine added was reduced and the ratio of iodine to sodium hydroxide in terms of normal concentration was 1:1.25. They also found that fructose was slightly oxidised at 17.5°, the amount of oxidation varying with concentration, amount of alkali present, time of reaction and temperature. Hinton and Macara attribute the discrepant results obtained by earlier workers to the use of impure samples of sugars.

Evans [1928] repeated Hinton and Macara's work on the oxidation of glucose at 17.5° and also made observations at 5°. He found that oxidation of glucose was complete in 45 minutes at 5° and obtained the same iodine value at both temperatures, namely 1.402 g. of iodine reduced per g. glucose. This is slightly lower than the theoretical value 1.410 obtained by Hinton and Macara. Evans obtained somewhat higher values than Hinton and Macara for the amounts of iodine reduced per g. fructose in spite of the lower

temperature, probably owing to the much longer time of reaction. From the oxidation of invert sugar by iodine at 5° Evans found 0.022 g. of iodine reduced per g. of fructose. Hinton and Macara determined the iodine value for fructose in solutions containing large amounts of fructose relative to glucose and deduced from their results that in invert sugar the value would be 0.012 g.

From the results obtained by these observers it seemed clear that before applying the iodimetric method to apple extracts a thorough investigation of the oxidation of fructose under carefully controlled experimental conditions was necessary, since by far the largest proportion of reducing sugar in the apple is fructose. Examination of the methods of purification of glucose used by Hinton and Macara and by Evans showed that the latter did not employ a high temperature for the final drying of the sample of glucose used. As it is difficult to remove the last traces of water from the sugars without applying heat it is possible that the slightly low values Evans obtained for the amount of iodine reduced per g. of glucose were due to insufficient drying. Since the oxidation of fructose by iodine is considerably affected by both temperature and time of reaction, it was thought that more consistent results would be obtained by using the longer time of reaction necessary for complete oxidation of glucose at a low temperature than by using the short time necessary at the temperature employed by Hinton and Macara. Small variations in either time or temperature would result in less difference in the amount of oxidation of fructose at a low than at a high temperature; for this reason Evans worked at a temperature of 5°. The oxidation by alkaline iodine of carefully purified samples of glucose and fructose has now been investigated at 1°.

It has also been necessary to study the effect of clearing and of the use of different deleading agents on the results obtained by the iodimetric method. In the preparation of apple extracts for estimation of sugars by the copper reducing method Haynes and Archbold [1928] found basic lead acetate and potassium oxalate satisfactory as clearing agents, although a correction of 3 % of the sugar estimated was necessary owing to the loss of sugar in the clearing process. They also found that reliable results could be obtained by using the uncleared solution, obtained by diluting either the expressed juice or an alcoholic extract after evaporation of the alcohol.

Preparation of samples of glucose and fructose.

A sample of glucose purchased as pure was recrystallised three times from alcohol and water and then dried *in vacuo* over phosphorus pentoxide for 4 months. It was then dried at 105° to constant weight (2–3 hours). An ash determination was carried out, and the rotatory power and copper reducing power of the sample found. The results were as follows:

$[\alpha]_D^{20}$ (10 % solution) observed 52.73°; calculated 52.74° Ash < 0.01 %

Copper reducing power. Weight of glucose taken 0.4056 g./100 cc.

Weight of glucose found 0.4055 g./100 cc.

Table I. *Effect of time of reaction on the oxidation of glucose by alkaline iodine at 1°.*

Amount of glucose present in each estimation = 0.0811 g.

Blank titration 38.05 cc. of 0.051 *N* sodium thiosulphate = 20 cc. iodine (0.1 *N* approx.).

Time of reaction in hours	cc. thiosulphate = iodine reduced	g. iodine reduced per g. glucose
1	17.55	1.395
1½	17.70	1.408
2	17.75	1.411
2½	17.75	1.411
3	17.70	1.408
4	17.75	1.411
5	17.70	1.408
6	17.75	1.411
7	17.75	1.411
17½	17.80	1.415

oxidation of glucose by alkaline iodine, but for many hours the amount is too small to be detected.

The iodine value for glucose, 1.410 g., was confirmed by further estimations on different amounts of glucose, the time of reaction being 2 hours. One set of results is shown in Table II. Hinton and Macara's result for glucose is thus

Table II. *Oxidation of glucose by alkaline iodine at 1°.*Blank titration 38.05 cc. of 0.051 *N* sodium thiosulphate = 20 cc. iodine.

Sample I			Sample II		
Weight of glucose in each estimation = 0.0643 g.			Weight of glucose in each estimation = 0.0794 g.		
No.	Thiosulphate = iodine reduced cc.	Iodine per g. glucose g.	No.	Thiosulphate = iodine reduced cc.	Iodine per g. glucose g.
1	14.05	1.412	1	17.35	1.409
2	14.10	1.416	2	17.35	1.409
3	14.10	1.416	3	17.35	1.409
4	14.05	1.412			
5	14.00	1.407			

Mean of 8 estimations 1.411.

confirmed for a temperature of 1°, and it seems probable that, as suggested above, Evans's sample of glucose was not quite dry.

The oxidation of fructose at 1° in solutions containing different ratios of fructose to glucose.

The ratio of fructose to glucose in the mature apple varies in general from about 2.5/1 to 4.5/1, but in a few cases ratios as high as 6/1 may occur. Having established the iodine value for glucose at 1° therefore, a series of estimations was carried out on solutions containing proportions of fructose to glucose ranging from 0/1 to 40/1 in order to determine the correct iodine value of fructose in the presence of glucose.

For this purpose a 0.3 % solution of glucose and a 4 % solution of fructose were prepared. 20 cc. of the glucose solution were taken for each estimation and quantities of fructose varying from 8 to 40 cc. All volumes were made

up to 100 cc., and the estimations carried out as described above. Two or more determinations were carried out for each ratio of fructose to glucose. The amount of iodine reduced by fructose was found by subtracting the amount reduced by glucose alone from the total amount reduced by a given solution. The results are shown in Table III. It will be seen that the iodine value for

Table III. *Oxidation of fructose by alkaline iodine at 1° in mixtures of glucose and fructose.*

Time of reaction 2 hours.

cc. of 0.5 *N* NaOH = 5.

Weight of glucose in each estimation = 0.06 g. approx.

Titration of glucose alone = 29.75 cc. of 0.043 *N* thiosulphate.

Ratio fructose glucose (approx.)	Fructose present g.	Thiosulphate = iodine reduced by fructose cc.	Iodine reduced per g. of fructose g.
1 : 1	0.0617	0.15	0.0134
2 : 1	0.1234	0.35	0.0157
3 : 1	0.1850	0.55	0.0163
4 : 1	0.2454	0.75	0.0169
5 : 1	0.3085	0.95	0.0170
6 : 1	0.3927	1.15	0.0162
7 : 1	0.4250	1.20	0.0156
8 : 1	0.4980	1.30	0.0144
9.5 : 1	0.5750	1.45	0.0139
11 : 1	0.6900	1.70	0.0136
24 : 1	1.5671	3.00	0.0106
30 : 1	1.9589	3.60	0.0102
40 : 1	2.7020	4.70	0.0096

fructose increases from 0.013 g. to 0.017 g. as the ratio of fructose to glucose increases from 1/1 to 5/1; and then decreases again slowly reaching a value of 0.0096 when the ratio is 40/1. Over the range of fructose/glucose ratios found in the apple the value is 0.016 to 0.017; 0.017 being the value for ratios 4/1 and 5/1. Since a difference of 0.001 in the iodine value of fructose makes only a negligible difference in the calculation of the amounts of fructose and glucose in their mixtures, the value 0.017 has been adopted for the whole range of fructose/glucose ratios in the apple. The value for equal amounts of the two sugars under these experimental conditions is 0.013 g., nearly the same as that suggested by Hinton and Macara for a temperature of 17.5° and rather lower than that found by Evans at 5°.

Effect of variations in time of reaction and proportion of alkali on the iodine value of a mixture of glucose and fructose at 1°.

Variations in the iodine value of fructose in the presence of glucose with alkali concentration and time of reaction were next studied, to define the precision necessary to obtain accurate results at 1°. Hinton and Macara showed that if the alkali exceeded the amount required for conversion of the iodine to hypoiodite and iodide, slightly low results were obtained for the iodine value of glucose at 17.5°. In Table V the small effect of excess alkali on the glucose has been neglected, so that the time change in amount of iodine

reduced by fructose is probably slightly greater than the recorded value. It will be seen in Tables IV and V that even at the low temperature of 1° the oxidation of fructose is markedly affected by differences in both amount of alkali and time of reaction.

It is thus essential not to exceed the time necessary for complete oxidation of glucose to gluconic acid by more than a few minutes before stopping the reaction, and also to regulate the alkali concentration so that the amount present is just sufficient to convert the iodine to hypoiodite. The results are shown in Tables IV and V.

Table IV. *Effect of variation in the time of reaction on the iodine value of fructose at 1° .*

Ratio fructose/glucose = 2.5/1.

Amount of glucose in each estimation = 0.06 g. (approx.).

Titration of glucose alone = 27.00 cc. of 0.044 *N* sodium thiosulphate.

Time in hours	Thiosulphate = iodine reduced by fructose (cc.)	Iodine reduced per g. fructose (g.)
2	0.50	0.0179
2½	0.60	0.0215
3	0.65	0.0232
5	0.90	0.0321
6	1.00	0.0358
17	1.60	0.0573

Table V. *Effect of varying the proportion of alkali on the iodine value of fructose at 1° .*

Ratio fructose/glucose = 2.5/1.

Amount of glucose in each estimation = 0.06 g. (approx.).

Blank titration 44.20 cc. of 0.044 *N* sodium thiosulphate = 20 cc. iodine.

Titration of glucose alone = 26.60 cc. of 0.044 *N* sodium thiosulphate.

cc. 0.5 <i>N</i> NaOH added	Thiosulphate = iodine reduced by fructose (cc.)	Iodine reduced per g. of fructose (g.)
4	Insufficient alkali for complete oxidation of glucose present	—
5	0.5	0.0174
6	0.6	0.0209

The application of the iodimetric method to the determination of reducing sugars in apple extracts.

In his work on the sugars in the apple Evans [1928] determined the amounts of fructose and glucose present by combination of the iodimetric method and Lane and Eynon's [1923, 1] method of estimating reducing sugars by copper reduction. The values for the two sugars were obtained by solving the simultaneous equations

$$C_1x - C_2y = \text{iodine value per 100 cc. of solution,}$$

$$K_1x - K_2y = \text{copper reducing power of 100 cc. of solution,}$$

where C_1 and C_2 are the g. of iodine reduced per g. of glucose and fructose respectively, and K_1 and K_2 the g. of cuprous oxide formed per g. of glucose

and fructose at the dilution used. Substitution of the more accurate values 1.410 and 0.017 for the g. of iodine reduced per g. of glucose and fructose respectively for those used by Evans makes a difference of less than 1 % in the amount of fructose, and about 0.0005 % in the amount of glucose calculated by solving the above equations. There is therefore no serious error in the results quoted by him. Solutions obtained by diluting the expressed juice of the apple and clearing with basic lead acetate and potassium oxalate were used for the estimations, and Evans stated that potassium oxalate and sodium acetate are not attacked by alkaline iodine (see p. 109).

The clearing of apple extracts with basic lead acetate and potassium oxalate has since been thoroughly investigated by Haynes and Archbold [1928] who found that in cleared solutions the results of sugar estimations were about 3 % lower than in uncleared solutions. They further showed that a similar loss of sugar occurred in clearing artificial mixtures of sugars, acid and pectin, of the same composition as the apple extract. In the estimation of total and reducing sugars by copper reduction clearing of the apple extracts is thus not necessary. Estimations in the dilute uncleared solution must, however, be carried out immediately, as hydrolysis of sucrose occurs on standing. This is not the case in cleared solutions, which remain unchanged for months—a distinct advantage during a series of routine estimations. A comparison between the iodine values of cleared and uncleared apple juices showed differences amounting to 10 %, the uncleared solutions giving the higher values. The clearing was carried out in dilute solution as described by Evans [1928, p. 4] and the uncleared juice was diluted to the same extent for estimation. 75 to 100 cc. of the dilute solutions (containing about 0.06 g. reducing sugar) were used for each estimation, and the reaction was carried out as described on p. 103, except that 25 cc. of iodine and 6 cc. $N/2$ NaOH were used. The volume of sugar solution was always made up to 100 cc. before adding the iodine and alkali. It should be pointed out that this solution is diluted to twice its volume for the determination of copper reducing power. The iodine values must therefore be halved for calculation of the amounts of fructose and glucose by means of the simultaneous equations. The results are shown in Table VI and point to the presence of substances oxidisable by iodine other than sugar in the uncleared extract, since clearing is known to cause a loss of only 3 % in the sugars.

Table VI. *The iodine values of cleared and uncleared apple juices.*

100 cc. of diluted solution used for each estimation.

Blank titration 50.6 cc. of 0.049 N thiosulphate = 25 cc. iodine.

Sample	Thiosulphate—iodine reduced (cc.)		Iodine reduced by 100 cc. of diluted solutions (g.)	
	Cleared juice	Uncleared juice	Cleared juice	Uncleared juice
1	25.65	28.25	0.1595	0.1757
2	21.00	28.80	0.1306	0.1792
3	25.80	27.90	0.1604	0.1736

Hinton and Macara [1927] have suggested the use of chloramine-T and potassium iodide as a weaker oxidising agent than alkaline iodine, for the determination of aldose sugars in cases where oxidisable material other than sugar is present, but it was found on trial that the results of estimations in cleared and uncleared solutions still showed a difference of 10 %. Hence no advantage is obtained by using chloramine-T instead of alkaline iodine for the determination of fructose and glucose in apples. Clearing is therefore essential if the iodimetric method is to be used satisfactorily, and an advantage if only copper reducing values are required, since it avoids the necessity of immediate estimation. The clearing process has in consequence been re-investigated to determine its effect on the iodine values of sugar solutions.

For this purpose the following solutions were prepared: fructose, 20 %; glucose, 8 %; malic acid, 4 %; pectin, 0.3 %. Equal amounts of the four solutions were mixed, giving a solution of these substances in the concentrations in which they occur in the apple. Mixtures were also made omitting the pectin, and omitting both the pectin and the malic acid, and diluting with water to obtain the appropriate concentrations. Two aliquots of each solution were diluted and neutralised in exactly the same way as the apple juice. One aliquot was cleared with basic lead acetate and potassium oxalate, and the iodine value of 100 cc. determined while the iodine value of the other was determined in 100 cc. of uncleared solution. The results are shown in Table VII. In every case a higher value was obtained in the cleared solution.

Table VII. *The iodine values of sugar solutions before and after clearing with basic lead acetate and potassium oxalate.*

Approximate concentrations of constituents in the diluted solutions used for estimations:

Solution F = Fructose 0.2 %.
 „ G = Glucose 0.08 %.
 „ M = Malic acid 0.04 %.
 „ P = Pectin 0.003 %.

Blank titration 51.35 cc. of 0.049 *N* thiosulphate = 25 cc. iodine.

Solution used	Thiosulphate = iodine reduced (cc.)		Iodine reduced by 100 cc. of solution (g.)	
	Uncleared	Cleared	Uncleared	Cleared
Sample 1				
F + G	17.70	18.08	0.1101	0.1124
F + G + M	17.65	17.90	0.1097	0.1114
F + G + M + P	17.75	18.00	0.1104	0.1120
Sample 2				
F + G + M	17.90	18.48	0.1114	0.1149
F + G + M + P	17.90	18.35	0.1114	0.1141
Sample 3				
F + G + M	16.50	16.80	0.1027	0.1045
F + G + M + P	16.48	16.85	0.1024	0.1050

It has been stated by Davis [1916], Deerr [1916] and Geerligs [1909] that the loss of sugar on clearing with basic lead acetate is chiefly a loss of fructose. In that case no marked decrease in the iodine value, similar to that found in

the copper reduction value, would be expected since the amount of oxidation of fructose is so small. The consistently high results for the iodine values found in the cleared solutions, however, suggest that some oxidation by iodine of the potassium oxalate occurs. Consequently the effect of iodine on potassium oxalate alone and in the presence of glucose was investigated. Varying amounts of oxalate alone were allowed to react with iodine under the normal experimental conditions, while in a second set of estimations the solutions were acidified and titrated immediately. In both cases some oxidation occurred, the differences in titration from the blank varying from 0.1 cc. to 0.8 cc. of 0.05 *N* sodium thiosulphate as the amount of oxalate increased from 0.5 cc. to 10 cc. In the presence of glucose the amount of iodine used was greater than that required for the complete oxidation to gluconic acid by an amount equivalent to 0.2 cc. of 0.05 *N* sodium thiosulphate per cc. of potassium oxalate, a similar amount to that found using oxalate alone. There is therefore a small but definite oxidation of oxalate under these conditions not affected by the presence of sugar, and the use of potassium oxalate as a deleading agent requires a correction of about 0.2 cc. to the titration obtained. Since a correction has also to be made to the values obtained by copper reduction it was decided to try other deleading agents.

The effect of different clearing and deleading agents on the iodine values of sugar solutions.

Solutions of fructose and glucose containing in some cases malic acid and pectin, were cleared with both normal and basic lead acetate and deleading with potassium oxalate, sodium carbonate and sodium phosphate (Na_2HPO_4). Colloidal ferric hydroxide, as suggested by Evans, was also tested but proved less convenient owing to the bulky nature of the precipitate obtained. The method adopted was similar to that already described for testing the effect of clearing and solutions of the same strengths were employed (see Table VII). With both normal and basic lead acetate potassium oxalate gave higher values for the iodine equivalents in the cleared than in the uncleared solutions. Sodium carbonate did not completely remove the lead from solutions cleared with normal lead acetate, and in the course of the iodimetric estimation a precipitate of lead iodide was obtained on the addition of acid to the reaction mixture [see also Meade and Harris, 1916]. With basic lead acetate as a clearing agent, the removal of lead appeared to be complete, but the filtrate was not clear, and the iodine equivalent was about 1.5 % too low in the cleared solution.

With sodium phosphate (Na_2HPO_4) and normal lead acetate a clear filtrate was obtained, but the solution was acid and required neutralising before the addition of alkaline iodine in order that the correct proportion of alkali should be present during the oxidation reaction. Using basic lead acetate a neutral filtrate was obtained, and the solution could readily be filtered at once. In addition, estimations of sugar, both iodimetrically and by copper reduction

showed that by using sodium phosphate as the deleading agent there was no loss of sugar in the clearing process. Norris and Brodie [1918] state that sodium phosphate completely removes lead and has no effect on the reducing power of sugar solutions, and Englis and Tsang [1922] found the smallest loss of sugar with this deleading agent.

Further trials with sodium phosphate as a deleading agent, using both normal and basic lead acetate as clarifying agents, were then carried out. The sugar solutions containing acid and pectin were cleared and deleading, and allowed to stand for various times before filtering. It was found that prolonged standing, 3 days, caused re-solution of some lead, since both with normal and basic acetate a precipitate of lead iodide appeared during the iodimetric estimation. The agreement between the results for shorter times of standing up to 16 hours was good in both cases. In view of the extra process of neutralisation required when normal lead acetate is used, and also the incomplete precipitation of malic acid by this salt, it was decided to continue the use of basic lead acetate for clearing the apple extract, but to use sodium phosphate instead of potassium oxalate as a deleading agent. The results of these trials are shown in Tables VIII and IX.

Table VIII. *The effect of clearing with normal and basic lead acetates, and of using different deleading agents on the iodine values of sugar solutions.*

Solutions as in Table VII.

Blank titration 57.4 cc. of 0.044 *N* thiosulphate = 25 cc. iodine.

Clearing agent	Solutions used	Thiosulphate = iodine reduced (cc.)		Iodine reduced per 100 cc. of solution (g.)	
		Uncleared	Cleared	Uncleared	Cleared
Deleading agent potassium oxalate.					
Sample 4					
Normal lead acetate	F + G	24.00	24.25	0.1330	0.1343
	F + G + M + P	24.05	24.45	0.1335	0.1354
Deleading agent sodium carbonate.					
Normal lead acetate	F + G	24.00	25.00	0.1330	0.1385*
	F + G + M + P	24.00	24.25	0.1330	0.1343*
Sample 5					
Basic lead acetate	F + G + M + P	22.38	22.05	0.1237	0.1222
Deleading agent sodium phosphate (Na ₂ HPO ₄).					
Basic lead acetate	F + G + M + P	22.38	22.40	0.1237	0.1241
Cleared with colloidal ferric hydroxide.					
	F + G	22.25	22.75	0.1233	0.1260
	F + G + M + P	22.30	23.00	0.1237	0.1274

* Lead iodide formed during the reaction.

Lane and Eynon [1923, 2] have objected to the use of sodium phosphate as it does not entirely remove alkaline earth metals, and their presence will affect the copper reduction of a sugar solution. This objection, however, does

Table IX. *The effect of time of standing on the iodine values of solutions cleared with normal and basic lead acetate and deleaded with sodium phosphate.*

Blank titration 57.0 cc. of 0.044 N thiosulphate—25 cc. iodine.
 Iodine values of 100 cc. of uncleared solutions 0.1327 g. ¹; 0.1282 g. ²

Time of standing	Thiosulphate—iodine reduced (cc.) (clearing agent)		Iodine reduced per 100 cc. solution (g.) (clearing agent)	
	Normal lead acetate	Basic lead acetate	Normal lead acetate	Basic lead acetate
Filtered at once	—	23.90	—	0.1324 ¹
2 hours	23.05	23.10	0.1277	0.1280 ²
Overnight	23.20	23.15	0.1285	0.1282 ²
3 days		Lead iodide formed		

not hold for apple extracts since the amounts of calcium present are too small to cause any error.

The use of sodium phosphate as a deleading agent in the clearing of apple extracts.

As the use of basic lead acetate and sodium phosphate had proved quite satisfactory for clarification of mixtures of sugars, malic acid and pectin, this method of clearing was applied to a series of extracts from stored apples. These extracts were prepared both by alcoholic extraction of the fresh tissue [Haynes and Archbold, 1928, p. 976] and by expression of the juice from previously frozen pulp [Haynes, 1925, p. 78]. The clearing method described by Evans was used, but a saturated solution of sodium phosphate was substituted for the potassium oxalate. 2–3 cc. of phosphate were added for each cc. of basic lead acetate solution. The solutions prepared from the alcoholic extractions were found to develop a yellow colour on standing, the colour becoming more intense as the time of standing was prolonged. No improvement was obtained by keeping in the dark instead of exposed to direct sunlight on the bench. The solutions prepared from the expressed juice remained quite colourless on standing. No colour appeared in either case if the solutions were deleaded with potassium oxalate. A comparison of the iodine values showed that the values for the coloured solutions which had been deleaded with sodium phosphate were considerably higher than those for the colourless solutions deleaded with potassium oxalate. Three sets of comparative values are shown in Table X. No correction has been made for the iodine reduced by the potassium oxalate; this correction would of course slightly increase the difference between the two sets of observations. Extracts prepared from very immature fruit were found to be coloured whichever deleading agent was used, and by solving the equations obtained by determining the iodine value and copper reducing power, negative values for fructose were obtained. These results show that in the coloured solutions iodine is reduced by some substance other than sugar.

Table X. *Iodine values of solutions prepared from alcoholic extracts of apple tissue cleared with basic lead acetate and delead with sodium phosphate and potassium oxalate.*

Solution	Solution delead with	
	Sodium phosphate Iodine reduced per 100 cc. (g.)	Potassium oxalate Iodine reduced per 100 cc. (g.)
1	0.1635	0.1513
2	0.1927	0.1722
3	0.1624	0.1465

Since the use of alcoholic extracts is preferable to the use of expressed juice [Haynes and Archbold, 1929] for sugar estimations, and the results obtained when sodium phosphate is used as the deleading agent require no correction for loss of sugars during clearing, the coloured solutions were boiled with charcoal to see if decoloration without loss of sugar was possible.

Table XI. *Iodine values of solutions prepared from alcoholic extracts of apple tissue cleared with basic lead acetate and sodium phosphate and decolorised by charcoal.*

Solution	Coloured solution	Decolorised solution
	Iodine reduced per 100 cc. (g.)	Iodine reduced per 100 cc. of original solution (g.)
1	0.1778	0.1566
2	0.1965	0.1621
3	0.1637	0.1491

Trials were made with animal charcoal and a sample of "suchar¹." The "suchar" was found to be much more efficient for decolorising the solutions and was used for all subsequent work.

The iodine values of the colourless solutions obtained by boiling with charcoal were next determined. 200 cc. of the neutral cleared, coloured solution were boiled with 0.5 g. of charcoal for 1 minute. The solution was filtered and the charcoal thoroughly washed with boiling water. The volume of filtrate was then made up to 250 cc., and the iodine value determined in the usual way. In every case a decrease in the iodine value was found as compared with the coloured solutions. Three sets of results are shown in Table XI.

In some cases one boiling was not sufficient to decolorise the solution, if this was so the solution was filtered and again boiled for 1 minute with fresh charcoal. This process was repeated until the solution was colourless. The iodine value decreased with each successive boiling until the solution was colourless, after which further boilings had no effect. It was found necessary to boil once more after the solution was apparently colourless in order to ensure a constant result. The effects of increasing the quantity of charcoal used and the time of boiling were tried, but no advantage was obtained in

¹ Suchar is a charcoal specially prepared for sugar refining by the British Suchar Processes, Ltd., and kindly supplied by them.

either case. The results are shown in Table XII. Tests were then carried out with invert sugar solutions to which sucrose had been added to see if adsorption occurred during the boiling process. No change was found in the iodine

Table XII. *Iodine values of a cleared extract of apple tissue after different times of boiling with varying amounts of charcoal.*

No. of boilings	Time of each boiling (mins.)	Wt of charcoal (g.)	Iodine reduced per 100 cc. of original solution (g.)
4 (colourless after 3rd boiling)	1	0.5	0.302
4 (left to stand $\frac{1}{2}$ hour before filtering off charcoal)	1	0.5	0.298
3	1	1.0	0.301
3 (colourless after 2nd boiling)	5	0.5	0.299

value after boiling once with charcoal, and after six boilings a loss of only 0.8 % was observed. The reducing power estimated by copper reduction however decreased 1 % after one boiling and 5 % after six boilings. This loss of sugar occurred in both total and reducing sugars and suggests that a slight adsorption of fructose and sucrose occurs during the boiling, while glucose is not affected. Consequently no change is found in the iodine value after one boiling with charcoal.

The iodine value and reducing sugars determined by copper reduction are shown in Table XIII.

Table XIII. *The effect of boiling with charcoal on the reducing power and iodine values of an invert sugar solution containing sucrose.*

No. of boilings	Reducing sugar (by copper reduction)		Sucrose g./100 cc.	Iodine reduced per 100 cc. (g.)
	g./100 cc.	g./100 cc.		
Unboiled	0.720	1.032	0.312	0.0753
1	0.713	1.026	0.313	0.0753
2	0.711	1.006	0.295	---
6	0.685	0.936	0.251	0.0747

The copper reducing power can be satisfactorily determined in the uncleared, or in the cleared but coloured solution, and the results combined with iodine values determined in the decolorised solution. The loss of reducing power during decoloration is therefore not a serious drawback to the use of sodium phosphate as a deleading agent. Since the cleared solutions darken considerably on standing, the effect of the more intense colour on the iodine value was next examined. An alcoholic extract of apple tissue was prepared and after evaporation of the alcohol the solution was diluted and cleared with basic lead acetate and sodium phosphate. The solution was colourless immediately after filtering, and an estimation of the iodine value was carried out at once, both with and without a preliminary boiling with charcoal. The

cleared solution was allowed to stand and again estimated after intervals of 3 and 8 months. More than one boiling was necessary to decolorise the solutions after long standing.

The results are shown in Table XIV.

Table XIV. *Iodine values of apple extract cleared and deleded with sodium phosphate estimated after different periods of standing both in coloured and decolorised solutions.*

No. of boilings required	Cleared coloured solutions estimated			Cleared decolorised solutions estimated		
	At once	After 3 months	After 8 months	At once	After 3 months	After 8 months
	—	—	—	1	2	4
Sample 1	0.2089	0.2089	0.2067	0.1796	0.1750	0.1767
Sample 2	—	0.1635	0.1625	0.1442	0.1448	—
Sample 3	0.1927	0.1907	—	0.1703	0.1739	—

No change was found in the iodine values of either the coloured or decolorised solutions after 8 months. Thus depth of colour does not in itself tend to increase the iodine value. In order to test the validity of the figures obtained by this method a comparison was made with iodine values of solutions deleded with potassium oxalate; since it was possible that the colourless solution obtained in this way might also contain the oxidisable material, estimations were carried out on solutions which had been boiled with charcoal in addition to the ordinary estimation. In this case the iodine value increased considerably during the boiling, and a solution boiled without the addition of charcoal showed an even greater increase. A solution of fructose and basic lead acetate and potassium oxalate showed the same increase in iodine value after boiling with or without the addition of charcoal, while the iodine value of a solution of potassium oxalate alone was unaffected by boiling. This increase appears to be due to some action of the excess of potassium oxalate on the sugars during boiling. Potassium oxalate is therefore unsuitable as a deleading agent for solutions prepared from immature apples. It has already been stated that such solutions are coloured even if deleded with oxalate, and the above results make it clear that decolorising by boiling is not practicable.

Table XV. *Iodine values, g. iodine reduced per 100 cc., of solutions deleded with sodium phosphate and potassium oxalate before and after boiling.*

Sample	Solution deleded with			
	Sodium phosphate		Potassium oxalate*	
	Not boiled	Boiled	Not boiled	Boiled
1	0.1635	0.1442	0.1513	0.1754
2	0.1927	0.1703	0.1722	0.2040
3	0.1624	0.1423	0.1465	0.1703

* No correction has been made for the oxidation of potassium oxalate. The results are therefore about 0.5 % too high.

The parallel experiments with extracts of mature apples (Table XV) using the two deleading agents showed that nearly the same result is obtained in the solution deleading with sodium phosphate after decoloration as in that deleading with potassium oxalate but not boiled. By varying the amounts of phosphate added during clearing it was found that the development of colour was largely dependent on the amount of phosphate present. Three solutions were prepared, one containing insufficient phosphate to remove all the lead, one with a slight excess of phosphate and one with a large excess. After standing for 3 months the first solution was still colourless, the second pale yellow and the third deeper yellow. The one containing excess lead was then completely deleading, and iodine values were determined on all three. Similar values were obtained for the two solutions which had stood with excess of phosphate, but the third gave a high value indicating a conversion of fructose to glucose in the presence of lead.

From the foregoing results it was concluded that for extracts prepared from mature apples satisfactory results could be obtained by using either potassium oxalate or sodium phosphate as the deleading agent, but that oxalate was inadmissible unless the cleared solution was colourless. The use of oxalate also has the disadvantage of requiring corrections both in the iodine and copper reduction values to give the correct results. The use of sodium phosphate requires no such corrections, but is more tedious since the additional process of decoloration is necessary. Since a good deal of work on immature fruit is in progress in this laboratory sodium phosphate has been adopted for routine work.

It has been found that the iodimetric method can be used satisfactorily for the estimation of much smaller amounts of sugar than those used in the work described here. It is now being employed in combination with the Hanes [1929] modification of Hagedorn and Jensen's method of sugar estimation for the determination of the small amounts of sugar in the developing apple. These results will be published shortly.

[*Note*, added 13th Feb.] The polarimetric method is not only tedious but there are indications that it is liable to give untrustworthy results in the case of apple extracts. Evans's [1928] results show quite close agreement between the actual polarimeter readings and those expected from the iodine and copper values of the solutions. Examination of his paper shows that he has assumed that the specific rotation of fructose is independent of concentration. If his data are recalculated using the formula relating specific rotation and concentration of fructose given by Vosburgh [1920, 1921] it is found that the polarimeter readings are uniformly lower than would be expected from the copper and iodine values. We have made observations which confirm this result, and there is thus some indication of the presence of a laevo-rotatory substance other than sugar in the cleared apple extract.

SUMMARY.

The oxidation of glucose and fructose by alkaline iodine at 1° and methods of preparation of apple extracts for iodimetric estimations have been investigated. Glucose is quantitatively oxidised to gluconic acid in two hours at 1° and some oxidation of fructose also occurs.

In mixtures of glucose and fructose the amount of iodine reduced per g. of fructose increases from 0.013 g. to 0.017 g. as the ratio of fructose to glucose increases from 1/1 to 5/1 and then decreases slowly as this ratio is further increased. The value 0.017 can be used for determination of fructose and glucose in apples by combination of the iodimetric and copper reduction methods, since the ratio of fructose to glucose in the apple is about 4/1.

The presence of oxidisable material other than sugar makes it necessary to clear the apple extracts before carrying out iodimetric determinations. Basic lead acetate with either sodium phosphate (Na_2HPO_4) or potassium oxalate as the deleading agent was found to give satisfactory results with extracts prepared from mature apples.

The loss of sugar during clearing and the slight action of iodine on potassium oxalate make corrections necessary for both the copper reducing value and the iodine value if oxalate is used as the deleading agent. If sodium phosphate is used there is no loss of sugar, but the cleared solution is yellow and still contains some non-sugar substance oxidisable by iodine. The solution can be decolorised by boiling with charcoal and satisfactory results obtained. Copper reduction determinations can be carried out on the yellow solution.

With very immature apples coloured solutions are also obtained when potassium oxalate is used, and these solutions cannot be boiled, as an increase in the iodine value occurs owing to some action of oxalate on the sugars. Sodium phosphate has therefore been adopted as the deleading agent for routine work.

REFERENCES.

- Baker and Hulton (1920). *Biochem. J.* **14**, 754.
 Cajori (1922). *J. Biol. Chem.* **54**, 617.
 Davis (1916). *J. Agric. Sci.* **8**, 7.
 Deerr (1916). *Int. Sugar J.* **18**, 166.
 Englis and Tsung (1922). *J. Amer. Chem. Soc.* **43**, 865.
 Evans (1928). *Ann. Bot.* **42**, 1.
 Geerligs (1909). *Int. Sugar J.* **11**, 276.
 Hagedorn and Jensen (1923). *Biochem. Z.* **135**, 46.
 Hanes (1929). *Biochem. J.* **23**, 99.
 Harding (1922). *J. Amer. Chem. Soc.* **44**, 1765.
 Haynes (1925). *Ann. Bot.* **39**, 77.
 ——— and Archbold (1928). *Ann. Bot.* **42**, 965.
 ——— (1929). *Reports of the Food Investigation Board for 1928-29*.
 Hinton and Macara (1924). *Analyst*, **49**, 2.
 ——— (1927). *Analyst*, **52**, 668.
 Judd (1920). *Biochem. J.* **14**, 255.
 Lane and Eynon (1923, 1). *J. Soc. Chem. Ind.* **42**, 132 T.
 ——— (1923, 2). *J. Soc. Chem. Ind.* **42**, 143 T.
 Meade and Harris (1916). *J. Ind. Eng. Chem.* **8**, 504.
 Norris and Brodie (1918). *Int. Sugar J.* **20**, 238.
 Vosburgh (1920). *J. Amer. Chem. Soc.* **42**, 1696.
 ——— (1921). *J. Amer. Chem. Soc.* **43**, 219.
 Willstätter and Schüdel (1918). *Ber. deutsch. chem. Ges.* **51**, 780.

XVI. BIOLOGICAL OXIDATIONS IN THE SUCCINIC ACID SERIES.

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THE oxidation of succinic acid can be accomplished by cells of very diverse types, ranging from the bacteria to those of mammalian muscle and brain. Early work on the course of this oxidation, by Batelli and Stern, Einbeck, and Dakin, showed that succinic acid was oxidised to fumaric acid and that this took up the elements of water to form *l*-malic acid. Hahn, Haarman and Fischbach [1929] demonstrated that in presence of muscle *l*-malic acid was further oxidised to oxaloacetic acid. In the case of bacteria it was clear [Quastel, 1924] that the main course of oxidation of succinic acid was through fumaric acid and pyruvic acid, oxaloacetic acid presumably being an intermediate step. The question, however, arose as to whether fumaric acid was oxidised directly or whether it was necessarily transformed to malic acid prior to oxidation. It was shown with bacteria, using the methylene blue technique [Quastel and Whetham, 1924], that malic acid was not a ready donor of hydrogen, a fact contrary to what would be expected if the oxidation of fumaric acid proceeded through malic acid. The evidence was sufficient to justify the opinion that fumaric acid might be oxidised directly. The discovery, however, that the donating powers of malic acid might be obscured by the accepting powers of the acid—presumably through the formation of fumaric acid which is a ready hydrogen acceptor—vitiates this conclusion, and later on the formulation of the theory of activation [Quastel, 1926] made it appear very unlikely that fumaric acid would be oxidised directly, an activated fumarate molecule, on the theory, tending to take up positive electricity (protons or hydrogen) rather than negative (oxygen). The question, therefore, was left quite open. Most tissues and organisms can bring about a change of fumaric acid to *l*-malic acid and can accomplish the oxidation of the latter acid, but neither of these facts constitutes evidence that the main line of oxidation of fumaric acid lies through *l*-malic acid. In the case of certain bacteria, for instance, fumaric acid gives rise (in presence of ammonium ions) to *l*-aspartic acid, an amino-acid capable of being oxidised by the bacteria in question. But it cannot be assumed from these facts that the main line of oxidation of fumaric acid, with these bacteria, lies through *l*-aspartic acid. The actual finding among the products of oxidation of succinate or fumarate

of any substance which is capable of further oxidation does not constitute evidence that this substance occurs on the main line of oxidation of the succinate or fumarate. Quantitative, in addition to qualitative, evidence is required in order properly to appraise the significance of any such substance.

An attempt is made in this communication to show, from a quantitative standpoint, that the biological oxidation of fumaric acid proceeds largely, if not entirely, through *l*-malic acid. This is done by comparing the rates of oxidation of fumaric and malic acids in presence of various organisms and tissues, by comparing the action of inhibitors on these oxidations and by measuring the malic acid produced from succinic acid. At the same time measurements have been made of the relative rates of oxidation of succinic, fumaric, malic and aspartic acids in presence of various tissues and organisms and of the influence these substances have on each other's oxidation. Results have been obtained showing very clearly how the organisms and tissues differ in significant ways from each other.

Of considerable importance to the problems of respiration is the inhibitory action of malonate on succinate oxidation, first found by the methylene blue method in the case of bacteria [Quastel and Whetham, 1925; Quastel and Wooldridge, 1928]. It will be shown that this inhibitory action applies not only to the oxidation of succinate by bacteria, but to that by muscle and brain tissue. The degree of inhibition varies widely, being greatest with muscle and brain. The reasons for this and the bearing of the results on the views held by Warburg are discussed.

EXPERIMENTAL.

Oxidation rates were measured in the Barcroft differential manometer, all experiments being carried out at 37°. Acids were used in the form of their sodium salts, the p_H of solutions being 7.4. The oxygen uptakes recorded are the net uptakes due to the substrates alone—the control oxygen uptake by the organism or tissue alone being subtracted from the uptake given by organism or tissue in presence of the substrate. On some occasions the same amount of organism or tissue was placed in each cup of the apparatus, the substrate under investigation being placed only in the right hand one. The oxygen uptake in such cases was that due to the substrate alone.

Phosphate buffer solution ($M/15$) was always present with the tissue, and substrates were present at an initial concentration of $M/15$. The total volume of solution in each cup of the apparatus was 3 cc.

The rates of oxidation of substrates were linear over the first 2 or 3 hours of the oxidation indicating that the initial concentration ($M/15$) used was sufficient to saturate the enzymes involved.

The various bacteria were prepared by 48 hours' growth either in tryptic broth or on nutrient agar plates, the organism in the first case being centrifuged and washed twice with 0.85 % saline, and in the second case being scraped off the plates, suspended in saline and then centrifuged and washed;

after washing, a homogeneous suspension of the organism in saline was prepared. 1 cc. of this suspension was used in each experiment. The organism was used as soon as possible after its preparation, its age being of great importance so far as the oxidations of fumarate, malate or aspartate are concerned. When not in use the organism was stored at 0°.

In the cases of muscle and brain, 0.5 g. of the tissue was always used. Muscle, after dissection from the animal, was cooled and minced. Brain was similarly treated, the whole brain in the case of the rabbit being used. With human brain only the grey matter of the cerebral cortex, after careful removal of the membranes and adhering blood vessels, was used.

In Table I in which most of the experimental results are set out, the

Table I. *Uptake of oxygen (at 37°) in mm.³ by organisms and tissues in presence of various substrates and mixtures of substrates, the initial concentration of each being M/15.*

Exp.	Organism or tissue	Succi- nate	Fuma- rate	l-Ma- late	l-As- partate	Succi- nate + fuma- rate	Succi- nate + l-ma- late	Succi- nate + l-as- partate	Succi- nate + malonate
1	<i>B. coli</i>	1456	698	770	710	1202	1185	1312	1108
2	"	1372	662	651	—	1064	924	—	—
3	" (few days old)	440	17	—	—	251	—	—	45
4	<i>B. acidi lactici</i>	1210	716	630	475	—	—	—	—
5	"	1072	—	—	—	842	889	806	—
6	"	210	5	—	—	123	—	—	37
7	<i>B. prodigiosus</i>	487	293	391	495	335	538	591	382
8	<i>B. protus</i>	1818	984	1035	1270	1445	1578	1525	1550
9	"	552	237	—	—	406	—	—	345
10	<i>B. pyocyaneus</i>	470	550	447	532	—	—	—	—
11	"	429	—	—	—	370	403	533	—
12	"	497	—	—	—	—	—	—	279
13	<i>B. alkaligenes</i>	358	75	155	257	242	287	575	108
14	"	541	—	—	—	415	479	1362	—
15	"	720	248	—	—	496	—	—	386
16	<i>B. subtilis</i>	443	—	—	352	—	—	1136	—
17	"	889	715	971	1342	—	—	—	710
18	<i>M. lysodeikticus</i>	728	584	416	242	632	718	1160	645
19	Rabbit muscle	302	—	—	92	—	—	544	—
20	"	900	45	477	400	528	640	1050	137
21	"	691	40	10	—	405	300	769	0
22	Rabbit brain	445	50	102	0	365	439	385	3
23	" (few days old)	410	41	21	—	—	—	—	37
24	Human brain (grey matter)	804	9	—	—	690	—	—	108
25	"	201	47	38	23	—	—	—	—

results of any one experiment are quantitatively comparable with each other, but not with those of a differently numbered experiment. The results given are representative of a large number of experiments.

RELATIVE RATES OF OXIDATION OF SUCCINIC, FUMARIC, MALIC
AND ASPARTIC ACIDS.

The rates of oxidation, as previously stated, are approximately linear over the first 2 or 3 hours. In most of the experiments a period of 2 hours was made the limiting time, and the actual oxygen uptakes of a number of substrates at the end of this period are a measure of the relative rates of oxidation of these substrates.

Now it follows that if fumaric acid can only be oxidised *via l*-malic acid, the velocity of oxidation of fumaric acid cannot be greater than that of *l*-malic acid for equivalent saturation concentrations of these substrates. A distinctly higher rate of oxidation for fumarate would indicate a separate line of oxidation.

The experimental results recorded in Table I show, on the whole, that the velocities of oxidation of fumarate and *l*-malate are of the same order, a much higher figure being recorded, however, in the case of a specimen of rabbit muscle for *l*-malate. It is an interesting fact that in certain cases the velocity of oxidation of fumarate exceeds that of *l*-malate. This is notably the case with *M. lysodeikticus* and it has been observed several times with brain tissue. The excess of oxidation, however, is usually small so that although this excess may point to some direct oxidation of fumarate, the effect may also be attributable to a secondary factor.

The fact that the oxidations of fumarate and *l*-malate are usually of the same order points to the rapid transformation of fumarate into *l*-malate and hence to the presence of fumarase in the organisms and tissues investigated. Experiment shows this to be the case.

The oxidation of aspartic acid is of interest. Aspartic acid may undergo (1) a direct oxidation, or (2) loss of ammonia, in presence of those organisms possessing the necessary enzymes, to form fumaric acid [Quastel and Woolf, 1926; Cook and Woolf, 1928] and oxidation *via l*-malic acid. If course (2) is the sole mode of oxidation followed, the velocity of oxidation of aspartic acid should not be greater than that of *l*-malic acid; if course (1) takes place the velocity may be greater. The results show that the strict aerobes *B. alkaligenes* and *B. subtilis* oxidise aspartate at a much greater rate than *l*-malate, whereas the facultative anaerobes, *B. coli*, *B. acidi lactici*, oxidise the amino-acid at about the same or a lesser rate.

The results suggest that the strict aerobes in question attack the amino-acid by course (1) and this is supported by the fact that neither of the organisms possesses the power of transforming *l*-aspartic acid into fumaric acid.

The relative rates of oxidation of succinate and fumarate vary very widely with different cells. The greatest variation is seen in the cases of muscle and brain tissue, with which the oxidation of fumarate is small compared with that of succinate. The reverse of this, when the oxidation of fumarate is seen to be even greater than that of succinate, occurs with *B. pyocyaneus*, an

observation entirely in harmony with the facts relating to the relative rates of fermentation of succinate and fumarate by this organism [Quastel, 1924].

The oxidations of fumarate and *l*-malate are very much more dependent on the age or condition of the organism than is the oxidation of succinate. Storing the organisms for a few days at 0° brings about a very marked decrease in the ability of an organism to oxidise fumarate or *l*-malate. It is this very high sensitivity of the oxidation to relatively slight changes in the cell which, doubtless, accounts for the fact that with muscle, even when used quite fresh, variable results are obtained for the oxidation of *l*-malate. To obtain reliable results with bacteria, these organisms must be used as soon as possible after their preparation. With *B. coli*, freshly prepared, the rate of oxidation of succinate is usually roughly twice that of fumarate; on storing the organism for a few days at 0° the rate of oxidation of succinate becomes as much as ten times that of fumarate—a ratio approximating to that found in muscle or brain tissue.

THE EFFECT ON SUCCINATE OXIDATIONS OF THE ADDITION OF FUMARATE, MALATE OR ASPARTATE.

It is now well known both from studies of bacteria and of muscle that the enzyme accomplishing the activation of succinate as a hydrogen donator will bring about the activation of fumarate as a hydrogen acceptor. It follows that fumarate is adsorbed at the succinate-activating enzyme and hence that it should compete with succinate for this enzyme. The addition, therefore, of fumarate to succinate (both at "saturation" concentrations) should bring about a diminution in the velocity of oxidation of the latter, the amount of diminution being dependent upon the relative degrees of adsorption. Experiment shows this to be the case. Usually it is found (see Table I) that the oxygen uptake of a mixture of succinate and fumarate is the average of that due to succinate alone and that due to fumarate alone, this being the case whether the oxidation of fumarate is small or large compared with that due to succinate. An exception to this rule has been found with muscle and the interesting question arises as to whether the adsorption of fumarate at the succinate enzyme is the same whatever the source of the enzyme. There is not yet, however, sufficient experimental evidence available to decide this point.

Incidentally the fact that fumarate inhibits succinate oxidation is in harmony with the hypothesis that the main course of oxidation of succinate is through fumarate. Were there a different means of oxidising succinate, it would have been anticipated that the addition of fumarate would have increased the rate of oxidation found in presence of succinate alone.

Since fumarase is present in the organisms and tissues under investigation, the effect of adding *l*-malate is virtually the same as adding an equivalent concentration of fumarate, for both give rise to the same equilibrium mixture. Experiment shows that the inhibiting action of *l*-malate is rather less than that

of fumarate, an effect presumably to be accounted for by the time factor involved in the equilibrium concentration of fumarate being formed from the malate. For instance *M. lysodeikticus* is slow compared with *B. coli* in transforming fumarate into malate, and accordingly we find that the degree of inhibition of succinate oxidation by malate is less with the former organism than with the latter.

Aspartate, in the presence of those organisms which can convert aspartic acid into fumaric acid, inhibits the oxidation of succinate. With those organisms which do not possess this power, however, aspartate increases the rate of oxidation found with succinate. Thus with *B. coli*, *B. acidi lactici* and *B. proteus*, which are organisms capable of forming *l*-aspartic acid from fumaric acid, the effect of aspartate is to inhibit succinate oxidation. Now, were the aspartate directly oxidised, instead of passing through fumarate and malate, the action of aspartate would be to increase the rate of oxidation in presence of succinate. With the strict aerobes *B. alkaligenes*, *B. subtilis* and *M. lysodeikticus* this actually occurs, there being a marked increase in the rate of oxidation on the addition of aspartate to succinate. This shows clearly the difference in mode of attack on the amino-acid between the strict aerobes and the facultative anaerobes.

B. prodigiosus seems to behave, to a small extent, in this respect like a strict aerobe.

The interesting fact emerges, however, with the strict aerobes, that the rate of oxidation of a mixture of aspartate and succinate is often greater than the sum of the individual rates. This does not always occur and the phenomenon depends very largely on the condition of the organism.

The same phenomenon applies to muscle tissue. Here again the addition of aspartate increases the rate of oxidation due to succinate, the new rate sometimes being greater than the sum of the individual rates. The effect is reminiscent of Needham's observation [1930] that a mixture of glutamic and aspartic acids gives rise to more succinic and malic acids than the sum of the acids produced from glutamic and aspartic acids individually.

With brain tissue, *l*-aspartate appears to have but little effect on succinate oxidation.

Both with muscle and brain tissue fumarate and malate inhibit succinate oxidations, the effects being rather less than with bacteria.

It is quite clear, as far as aspartate oxidation is concerned, that, both with muscle tissue and the strict aerobes, the line of oxidation is not mainly through fumarate and malate. With the facultative anaerobes, on the other hand, this course would appear to be the predominant one. The alternative would be that for these organisms alone the aspartate molecule *per se* is highly adsorbed at the succinate enzyme—a hypothesis for which there is as yet no evidence.

THE ACTION OF MALONATE.

It would be anticipated that if fumarate underwent a direct oxidation, this would take place at the enzyme which is known to activate the fumarate molecule, *i.e.* at the succinate dehydrogenase. Though the electrical theory of activation does not favour the possibility of the activated fumarate molecule taking up negative electricity (*i.e.* of becoming directly oxidised), there is no definite evidence so far that this does not in fact occur. Now it has been shown that malonate competes with succinate for the enzyme capable of activating the latter, and Cook [1930] has recently shown that this inhibitory action of malonate on succinate oxidation applies aerobically, as well as anaerobically, to *B. coli*. There is little doubt that the competing action of malonate with succinate for the latter dehydrogenase is a general phenomenon—applying to muscle and brain tissue (see Table I) as well as to various classes of bacteria.

It would be expected, therefore, that if fumarate is oxidised at the succinate enzyme, where it is known to be adsorbed, the presence of malonate would inhibit its oxidation by competition for the enzyme.

The action of malonate on fumarate oxidation provides, therefore, a good test of the possibility that fumarate undergoes an oxidation at the succinate enzyme.

Experiment shows that the action of malonate both on fumarate or malate oxidation is either nil or very small.

For instance with *B. coli*

Fumarate (<i>M</i> /37)	took up 423 mm. ³ O ₂ in 2 hrs.		
Fumarate (<i>M</i> /37) + malonate (<i>M</i> /15)	„	430	„
<i>l</i> -Malate (<i>M</i> /37)	„	427	„
<i>l</i> -Malate (<i>M</i> /37) + malonate (<i>M</i> /15)	„	400	„

The fact that malonate has either little or no effect on fumarate and malate oxidations explains the wide variations observed in the degrees of inhibition by malonate on succinate oxidation with various organisms and tissues. In the case of those cells with which the rate of fumarate oxidation approaches that of succinate, malonate has a relatively small effect; for, in spite of the large effect of malonate on the succinate-fumarate reaction, fumarate is still produced in sufficient quantity to allow a relatively rapid uptake of oxygen. (It is worthy of note that the succinate-fumarate reaction requires only one atom of oxygen, whilst the complete oxidation of fumarate requires six atoms.) On the other hand, in the case of those organisms with which the fumarate oxidation is slow compared with that of succinate, malonate has a large effect, the action of the malonate on the succinate-fumarate reaction being predominant. This is the case with muscle and brain tissue with which the fumarate oxidation is normally small, or with organisms which have been stored so long or treated in such a way that the fumarate and malate oxidations have disappeared. Here the malonate inhibition ap-

proaches 90 % or an even larger value. With organisms, however, such as *M. lysodeikticus* or *B. pyocyaneus* where fumarate oxidation is relatively high the malonate inhibition may approach only 30 %.

The small or negligible action of malonate on fumarate oxidation is, definitely, evidence against the view that fumarate might undergo a direct oxidation at the succinate enzyme.

THE ACTION OF OXALATE.

If the oxidation of fumarate proceeds largely through *l*-malate it follows that an agent which inhibits malate oxidation will also inhibit fumarate oxidation. Such an agent is oxalate. The following results were obtained with *B. pyocyaneus*, all the substrates being initially present at a concentration of *M*/15 and the oxalate as the potassium salt.

<i>l</i> -Malate	took up 551 mm. ³ O ₂ in 90 mins.		
<i>l</i> -Malate + oxalate	„	330	„
Fumarate	„	600	„
Fumarate + oxalate	„	315	„
Succinate	„	547	„
Succinate + oxalate	„	409	„

It will be seen that oxalate exercises an inhibitory action on both malate and fumarate oxidations—the inhibition being greater than on that of succinate. Oxalate has not a large inhibitory action in the succinate-fumarate reaction [Quastel and Wooldridge, 1928] but it is to be expected it will appreciably inhibit the total oxidation of succinate, since the latter passes through fumarate and malate.

The fact that the inhibitions of fumarate and *l*-malate oxidations by oxalate are of the same order of magnitude is clearly evidence in favour of the view that fumarate oxidation proceeds *via l*-malate.

It is worthy of note that toluene, which eliminates the oxidation of fumarate by *B. coli* [Cook, 1930], also eliminates the oxidation of *l*-malate by this organism.

OXIDATION OF SUCCINATE TO *l*-MALATE.

Summing up the evidence so far it appears:

- (1) the velocities of oxidation of fumarate and *l*-malate are usually of the same order of magnitude;
- (2) malonate, which inhibits the succinate-fumarate reaction, has little action on the oxidation of either fumarate or *l*-malate;
- (3) oxalate inhibits the oxidation of fumarate and *l*-malate to about the same extent, the inhibition being greater than that observed with succinate.

Though this evidence, on the whole, is in favour of the view that the oxidation of fumarate proceeds largely through *l*-malate there is as yet no direct evidence that succinate does not suffer another oxidation (*e.g.* direct to *dl*-malate) besides that of proceeding, in the first place, to fumarate.

This evidence may be secured from a quantitative study of the oxidation of succinate in presence of those tissues or organisms with which the oxidation of fumarate or malate is small or nil. In presence of such cells, one atom of oxygen should be taken up per molecule of succinate forming an equilibrium mixture of fumarate and *l*-malate in the ratio of 1:3. Thus the uptake of 1 g.-atom of oxygen should give rise to $\frac{3}{4}$ g.-mol. of *l*-malate. If succinate suffered an oxidation other than that through fumarate, the uptake of 1 g.-atom of oxygen would lead to less than $\frac{3}{4}$ g.-mol. *l*-malate. If, for instance, it proceeded first to *dl*-malate, and this to a mixture of *d*-malate, *l*-malate and fumarate the final rotation observed would not only be smaller than if fumarate were the first step but of the opposite sign.

For experiment, succinate at an initial concentration of $M/15$ was oxidised in presence of (1) muscle tissue, with which the fumarate oxidation was very small compared with that of succinate; (2) a suspension of *B. coli* which had been treated with toluene so as to eliminate the oxidation of fumarate.

The oxidation was carried out in the Barcroft apparatus and after a certain period the oxygen uptake was read, and the solution in the Barcroft cup made up to 5 cc. with water. 1 cc. glacial acetic acid was added and the whole mixed with 10 cc. 11.2 % ammonium molybdate solution. After filtering or centrifuging, the clear solution was examined polarimetrically. Using the mercury green line a rotation of 1° (2 dm. tube) was equivalent to 9.65 mg. *l*-malic acid. The experimental results were as follows.

(1) *With muscle tissue:*

Oxygen uptake at 37° and 760 mm.	357 mm. ³
Rotation observed	0.30°
Rotation calculated on the basis of succinate being oxidised entirely through fumarate... ..	0.30°

(2) *With B. coli treated with toluene:*

Oxygen uptake at 37° and 760 mm.	1222 mm. ³
Rotation observed	1.06°
Rotation calculated	1.02°

These results clearly indicate that if succinate normally undergoes some other oxidation, besides that of proceeding through fumarate, the amount of this oxidation must be exceedingly small.

The entire evidence is now in support of the view that the normal course of biological oxidation of succinate lies largely, if not entirely, through fumarate and *l*-malate.

WARBURG'S RESPIRATORY ENZYME.

The inhibition of succinate oxidation by malonate in presence of the intact cell bears upon the views held by Warburg of the "respiration enzyme" of the cell. These views are best expressed in his own words [Warburg, 1930] "...if different oxidases occur in an extract of one kind of cell they are not

enzymes which were preformed in the living cell, but transformation and decomposition products of a substance uniform in life. Uniformity of respiration enzyme and multitude of oxidases in the extracts do not constitute a contradiction."

Presumably, then, on this view the system responsible for the oxidation of succinate in the intact cell is identical with that which is responsible for the oxidation of fumarate, or lactate, or (say) *p*-phenylenediamine. This view leaves no room for the existence of specific dehydrogenases in the intact cell and supposes that the effects of treatment, or extraction, of a cell, on the various oxidations, are not to eliminate certain dehydrogenases but to affect a common oxidase in such a way that the less "sensitive" substances are no longer oxidised whilst the more sensitive are still attacked.

That this view cannot be true follows not only from the fact that with the bacteria the dehydrogenases are selectively poisoned [Quastel and Wooldridge, 1927], but from quantitative evidence with the intact cell. It was shown for instance that the oxidase systems for succinate and glucose differed from each other. Were there a common oxidase for these substances in the intact cell, then the rate of oxidation of a mixture of the substances at their saturation concentrations would not be greater than the higher of the individual rates. Experiment showed that the oxidation rate was the sum of the individual rates.

In this communication it has been shown that with *B. alkaligenes*, *B. subtilis* and *M. lysodeikticus* the velocity of oxidation of a mixture of succinate and aspartate is greater than either of the individual rates—a fact showing the presence of two distinct activating systems in the intact cell.

Attention, however, might be drawn, in this connection, to the inhibiting action of malonate. It has been shown previously by the methylene blue method that this substance acts reversibly on *B. coli* [Quastel and Wooldridge, 1928]. Moreover malonate does not injure the cell, for the organism is capable of prolific growth in presence of it.

Now it would be expected, if succinate and *p*-phenylenediamine are oxidised by the same system in the intact cell, that malonate which inhibits succinate oxidation would also inhibit the *p*-phenylenediamine oxidation. Experiment shows that this is not the case.

Thus, with brain tissue (0.5 g.):

p-phenylenediamine (21 mg.) took up in 2 hrs. 1206 mm.³ O₂.

p-phenylenediamine (21 mg.) + malonate (*M*/15) took up in 2 hrs. 1201 mm.³ O₂.

p-phenylenediamine (21 mg.) + oxalate (*M*/15) took up in 2 hrs. 1132 mm.³ O₂.

Neither malonate nor oxalate appreciably inhibits *p*-phenylenediamine oxidation under conditions such that succinate oxidation is markedly inhibited by malonate.

The conclusion is clear that, for a consistent interpretation of the results of biological oxidations, it is necessary to picture the existence in the intact cell of distinct active centres, or dehydrogenase systems, each concerned with the activation of a certain type of hydrogen donator. Possibly, as already suggested [Quastel and Wooldridge, 1927], the process of treating or extracting a cell alters the activating range of each centre, but that more than one distinct activating centre exists in the intact cell there can be no question. The oxidase which is concerned with the activation of molecular oxygen is quite distinct from the systems already referred to. Whether there is one such oxidase or a number in the intact cell is still a problem for further investigation.

SUMMARY.

1. It has been shown from quantitative evidence that the normal course of biological oxidation of succinate proceeds largely, if not entirely, through fumarate and *l*-malate. The evidence results from

(a) a comparative study of the oxidations of fumarate and *l*-malate in presence of various organisms and tissues;

(b) a comparison of the action of malonate and of oxalate on the oxidation of fumarate and *l*-malate;

(c) measurements of *l*-malic acid formed from succinic acid; the calculated quantities are in close agreement with those observed.

2. Fumarate and *l*-malate inhibit the oxidation of succinate in presence of various bacteria and of muscle and brain tissues.

3. It is shown that the strict aerobes, *B. alkaligenes*, *B. subtilis* and *M. lysodeikticus*, and also muscle tissue, attack *l*-aspartate in a different manner from the facultative anaerobes, *B. coli*, *B. proteus* and *B. acidi lactici*. The latter oxidise the acid through fumaric and *l*-malic acids.

4. Malonate not only inhibits the oxidation of succinate by bacteria but also that by muscle and brain tissues, the degree of inhibition being greatest with these tissues. It is shown that the wide variation with different cells in the degrees of inhibition by malonate is associated with differences between velocities of oxidation of fumarate and those of succinate.

5. Neither malonate nor oxalate inhibits the velocity of oxidation of *p*-phenylenediamine by brain tissue.

6. These results are discussed in relation to Warburg's views on the "respiration enzyme" of the cell.

Our thanks are due to the Medical Research Council for a grant to this laboratory in aid of equipment and for a whole-time grant to one of us (A. H. M. W.). We also wish to express our appreciation of the very able assistance afforded us by Mr A. Dignam.

REFERENCES.

- Cook (1930). *Biochem. J.* **24**, 1538.
— — and Woolf (1928). *Biochem. J.* **22**, 474.
Hahn, Haarman and Fischbach (1929). *Z. Biol.* **88**, 587.
Needham (1930). *Biochem. J.* **24**, 208.
Quastel (1924). *Biochem. J.* **18**, 365.
— — (1926). *Biochem. J.* **20**, 166.
— — and Whetham (1924). *Biochem. J.* **18**, 519.
— — — (1925). *Biochem. J.* **19**, 525.
— — and Wooldridge (1927). *Biochem. J.* **21**, 148, 1224.
— — — (1928). *Biochem. J.* **22**, 689.
— — and Woolf (1926). *Biochem. J.* **20**, 545.
Warburg (1930). *Bull. Johns Hopkins Hospital*, **46**, 341.

XVII. A STUDY OF THE MECHANISM OF THE DEGRADATION OF CITRIC ACID BY *B. PYOCYANEUS* (*PSEUDOMONAS PYOCYANEA*).

II. ACTION OF *B. PYOCYANEUS* ON SUCCINIC ACID.

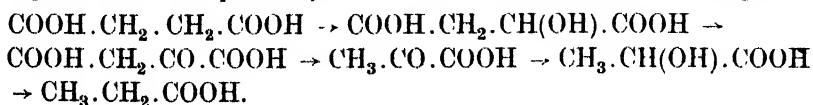
BY THOMAS KENNEDY WALKER, VIRA SUBRAMANIAM, HOWARD BRAITHWAITE STENT AND JACK BUTTERWORTH.

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IN Part I [Butterworth and Walker, 1929] it was shown that the products of the action of *B. pyocyaneus* on ammonium citrate include malonic acid and a small quantity of succinic acid, and it was suggested that the latter arises by simultaneous decarboxylation and dehydrogenation of two molecules of the malonic acid in presence of a hydrogen acceptor. Pyruvic acid, which might well be derived from the acetone also produced in the ammonium citrate cultures, could exercise such a function, being thereby converted to lactic acid or, under certain conditions, to propionic acid. Support for this hypothesis is to be found in the experiments of Aubel and Cambier [1922], who detected lactic acid in cultures of *B. pyocyaneus* on solutions of the salts of both citric and pyruvic acids. Further, as will be evident from results described in the course of the present communication, the fact that propionic acid has been shown to be an end-product of the action of *B. pyocyaneus* on ammonium succinate [Aubel, 1921; Quastel, 1924] supports the same view. We have also obtained additional experimental evidence in favour of such a possibility by establishing the fact that addition of small quantities of acetone to cultures of *B. pyocyaneus* on calcium acetate media is followed by the formation therein of lactic acid.

In view of the occurrence of succinic acid in the citrate cultures, and of the fact that propionic acid is formed by the growth of *B. pyocyaneus* on succinate media, it was considered of interest to make a closer study of the behaviour of succinic acid under the attack of this organism. It was considered unlikely that the propionic acid originates in the simple decarboxylation of succinic acid under the influence of the bacillus, as suggested by Aubel, since such a view is not in harmony with the known facts of organic chemistry, and its formation was regarded as more probably due to the following series of changes:



In the comprehensive work of Van Niel [1928] on the chemistry of the propionic acid bacteria, the formation of propionic acid from both pyruvic acid and lactic acid was verified beyond doubt.

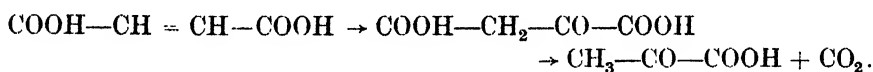
Adopting the above tentative scheme as a basis of enquiry, search for the presence of malic acid was made in cultures of *B. pyocyaneus* on ammonium succinate, and its formation was demonstrated in several experiments after fermentation had been in progress for about 9 days. The acid was detected by Denigès' mercuric acetate reagent [1900] and in subsequent experimental fermentations it was isolated and examined in detail, and, in every instance, it was found to consist of a mixture of the *dl*- and *l*-forms. Since *B. pyocyaneus* does not effect racemisation of the active forms of malic acid, and since we have proved by control experiments that *l*-malic acid is unaltered by the treatment adopted for its isolation from the cultures, there remain only two possible sources from which the *dl*-malic acid could have been derived, namely, (*a*) from the succinic acid by the action of activated hydrogen peroxide, for *B. pyocyaneus* is known to contain a peroxidase system, and (*b*) from oxaloacetic acid by symmetrical reduction. We regard (*a*) as the more probable alternative, for biological reduction has usually been observed to yield asymmetric compounds when structure has permitted optical activity and, further, the fact that *B. pyocyaneus* is rich in catalase is not an argument against (*a*), since it has been demonstrated clearly [Chodat and Pasmanik, 1907; Thurlow, 1925] that a partition of hydrogen peroxide between catalase and peroxidase occurs when both enzymes are present.

In order to throw light on the source of the *l*-acid the bacillus was then cultivated on media in which *dl*-malic acid constituted the sole source of carbon and, in the several experiments undertaken, such media invariably developed dextrorotatory properties. They also gave positive reactions to the test for pyruvic acid devised by Quastel [1924] and were free from lactic acid. Hence it follows that the *l*-malic acid obtained by the fermentation of ammonium succinate is not derived through resolution by the bacillus of preformed *dl*-malic acid, but must have its genesis in the asymmetric addition of water to fumaric acid arising from the dehydrogenation of succinic acid. In this connection it is to be noted that Stent, Subramaniam and Walker [1929] have recently shown that succinic acid yields a mixture of *dl*- and *l*-malic acids when submitted to the attack of *Aspergillus niger*, and that the latter organism renders solutions of *dl*-malic acid dextrorotatory, whilst Challenger and Klein [1929] have established the fact that the same strain of this mould produces high yields of *l*-malic acid from potassium fumarate, whence it would appear that the degradation of succinic acid is achieved in precisely the same manner by both *B. pyocyaneus* and *A. niger*.

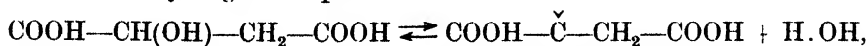
Quastel [1924], Quastel and Whetham [1924] and Quastel, Stephenson and Whetham [1925], in the course of studies of the reactions induced by resting bacteria have considered the cases of the equilibria which are set up (*a*) between fumaric acid and *l*-malic acid in the presence of resting *B. coli*, and (*b*) between

succinic acid and fumaric acid in the presence of methylene blue and resting *B. pyocyaneus*. Their experimental results are later interpreted as special cases of the general theory of the mechanism of oxidation and reduction *in vivo*, postulated by Quastel [1926], in which the breakdown of succinic acid by resting bacteria is assumed to proceed by initial formation of an activated form of fumaric acid, symbolised as $\text{COOH}-\text{CH}_2-\overset{\vee}{\text{C}}-\text{COOH}$, and regarded as identical with the activated form into which it is assumed fumaric acid is first converted under similar enzymic influence. This explanation is an elaboration and to some extent a modification of Wieland's dehydrogenation hypothesis, and if it also applied to the behaviour of succinic acid when submitted to the attack of actively growing *B. pyocyaneus* under aerobic conditions, we should expect to obtain products identical with those which arise by the action of this organism on fumaric acid. Such, however, is but partially the case for, as we have just seen, the breakdown of the succinic acid in this instance is largely achieved by what appears to be a peroxidase reaction (leading to the formation of racemic malic acid), which proceeds simultaneously with the dehydrase and fumarase reactions affording respectively fumaric acid and thence *l*-malic acid.

According to Quastel [1924] the growth of *B. pyocyaneus* on media containing salts of fumaric acid is very vigorous and malic acid cannot be detected among the products, of which the first to be identified is pyruvic acid. Further, Quastel and Whetham [1924] found malic acid to be incapable of donating hydrogen to methylene blue in the presence of the resting organism. As a result of these observations they concluded that the conversion of fumaric acid to pyruvic acid probably proceeds through the intermediate formation solely of oxaloacetic acid

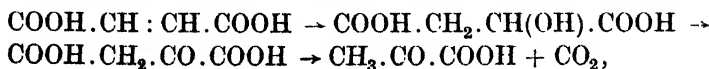


In a later paper, however, Quastel [1926] brings forward evidence which vitiates this conclusion, for he shows that the effect of an activating field on malic acid is to produce a certain amount of activated fumaric acid which functions as a hydrogen acceptor



in consequence of which he states that malic acid, after activation, behaves both as a hydrogen acceptor and donator, and considers that probably the donating properties are obscured in the experiments with *B. coli* by the accepting powers.

In our view the formation of *l*-malic acid in cultures of *B. pyocyaneus* on succinate media which, as we have demonstrated, can only have taken place by asymmetric addition of water to fumaric acid arising by preliminary dehydrogenation of the succinic acid, renders it more probable that the pyruvic acid produced in cultures of *B. pyocyaneus* on fumarates arises through the changes:



than by the direct oxidation of fumaric acid to oxaloacetic acid. This opinion must not be construed as belief on our part that direct oxidation of this type cannot take place, for Quastel's activation theory [1926] indicates a reasonable mechanism by which such a change could, in certain cases, be effected.

EXPERIMENTAL.

The strain of *B. pyocyaneus* employed was that used in Part I of this investigation, namely, Fildes III, No. 1999, from the National Collection of Type Cultures, Lister Institute. The substances under investigation were submitted to its action in Ringer's inorganic salt solution, the various media being adjusted to p_H 7.4 prior to inoculation.

Enzymic conversion of acetone to lactic acid.

Forty flasks of 500 cc. capacity were each charged with 5 g. of calcium acetate (Kahlbaum) and 1.0 g. of ammonium nitrate in 250 cc. of Ringer's solution. After sterilisation, 0.5 g. of pure acetone was delivered from a sterile pipette into each flask, followed by an inoculum of 1 cc. of a 24 hours old culture of the organism on the same medium. Incubation was conducted for 3 days at 25°, after which the contents of the flasks were combined and concentrated somewhat by distillation. The distillate contained no acetone. The residue was evaporated to dryness on the steam-bath. Preliminary tests having indicated that this material contained calcium lactate, a portion (20 g.) was distilled with 100 cc. of sulphuric acid (50 % concentration) until 30 cc. of distillate had passed through the ice-cooled condenser. The acetic and formic acids in this distillate were neutralised with sodium hydroxide and the solution was then distilled through the ice-cooled condenser until 20 cc. of distillate were obtained. This gave a strongly positive test for acetaldehyde by Rimini's reaction [1904]. It was mixed with a solution of *p*-nitrophenylhydrazine (5 g.) in acetic acid (40 cc. of 50 % concentration) and the precipitate was washed, dried and recrystallised from alcohol; M.P. 128° alone or in admixture with an authentic specimen of acetaldehyde *p*-nitrophenylhydrazone (M.P. 128°). Yield, 0.8 g. (found, N, 23.8; calc. N, 23.46 %).

Another portion (22 g.) of the original dry residue presumed to contain calcium lactate was heated with 50 % sulphuric acid as before, and the distillate was neutralised with sodium hydroxide and evaporated to dryness. The residue was extracted several times with methyl alcohol in order to remove sodium acetate, and the residual small quantity of insoluble solid material was taken up in 10 cc. of water and treated with 2 g. of lead nitrate dissolved in 10 cc. of water, when a precipitate formed and was removed. It was washed with cold water and recrystallised from hot water, yielding 0.8 g. of pure lead formate (found, Pb, 69.4; calc. 69.7 %).

A control experiment in which pure calcium lactate was distilled with sulphuric acid gave acetaldehyde and formic acid as above. In a blank experi-

ment no trace of lactic acid was detected in a solution of pure calcium acetate fermented in the absence of acetone.

Detection of malic acid in cultures of B. pyocyaneus on ammonium succinate.

The most delicate test for the detection of malic acid is that of Denigès [1900], and it was found that this could be combined with the use of 2 : 4-dinitrophenylhydrazine whereby a means was provided for obtaining quickly a rough approximation of the concentration of malic acid in a solution containing only a trace. Thus, dilute aqueous solutions were made up containing respectively 0.1, 0.05, 0.033, 0.025 and 0.02 % of malic acid. 25 cc. of each solution were treated with 3 to 4 cc. of Denigès's mercuric acetate solution, then boiled and filtered. Slight excess of a 2 % potassium permanganate solution was added to each, drop by drop, whilst the solutions were maintained at the boiling point. The precipitates of the basic mercury salt of oxaloacetic acid which were thus obtained were allowed to settle in test-tubes, the clear supernatant liquid was decanted and the precipitates were then each treated with 5 cc. of a saturated solution of 2 : 4-dinitrophenylhydrazine hydrochloride in 2*N* hydrochloric acid. This gave rise to slight precipitates of yellow crystalline material in all cases in which the malic acid had been present originally at a concentration of 0.025 % or higher. These crystals consist probably of a mixture of the 2 : 4-dinitrophenylhydrazones of pyruvic acid and acetaldehyde, since treatment of the basic mercury salt of oxaloacetic acid with cold dilute hydrochloric acid yields a solution which smells strongly of acetaldehyde, and which gives a positive reaction for pyruvic acid when treated with an alcoholic solution of guaiacol and strong sulphuric acid (Quastel's test [1924]). The results of the above-described experiments may be tabulated thus:

% concentration of malic acid in solution	0.1	0.05	0.033	0.025	0.020
Volume (cc.) of 2 % KMnO_4 solution equivalent to 25 cc. of malic acid solution	0.56	0.30	0.20	0.15	0.10
Quantity of 2 % KMnO_4 solution added (drops)	12	9	7	5	4
Precipitate	+ + +			+	+
Action of 2 : 4-dinitrophenylhydrazine	Yellow precipitate and golden opacity			Golden opacity. Precipitate on standing	Opalescence only

This information was then utilised in subsequent experiments in which it was sought to prove the presence of malic acid.

Ammonium succinate (30 g.) was dissolved in Ringer's solution (1500 cc.) and this mixture was distributed equally between 6 flasks of 500 cc. capacity. After sterilisation, inoculation was performed by addition to each flask of 1 cc. of an actively fermenting culture on the same medium, and incubation was conducted at 34°. At intervals of 7 hours portions of 25 cc. were withdrawn

and tested with 2 % KMnO_4 solution in the presence of mercuric acetate solution at the boiling point. After 3 days this test for malic acid was positive, whereupon 700 cc. of the culture solution were treated with these reagents and yielded 1.5 g. of the characteristic basic mercury salt of oxaloacetic acid which, on subsequent distillation with 5 g. of sodium iodide in 25 cc. of water, gave traces of acetaldehyde (Rimini's test). After 11 days the remaining contents of the culture flasks were combined and treated as above, when 18 g. of moist mercury salt were obtained. This was distilled with sodium iodide (30 g.) in water (150 cc.) and the well-chilled distillate was conducted beneath the surface of a solution of 2 : 4-dinitrophenylhydrazine hydrochloride (250 cc.) prepared according to the particulars given by Brady and Elsmie [1926]. The resultant precipitate (0.6 g.) was collected, washed, dried and recrystallised several times from hot ethyl alcohol, when it formed glistening orange-yellow crystals, m.p. 162° . This melting point was not depressed by admixture with a pure specimen of acetaldehyde 2 : 4-dinitrophenylhydrazone (found, by micro-method: N, 24.5; calc. N, 25.0 %).

Isolation of dl-malic acid together with l-malic acid from cultures of B. pycocyaneus on ammonium succinate.

Exp. 1. 2500 cc. of a 2 % solution of ammonium succinate in Ringer's solution, after adjustment to p_{H} 7.4, were equally distributed between 10 flasks of 500 cc. capacity. After sterilisation these were inoculated at the rate of 1 cc. per flask with an active culture which had grown for 24 hours on a medium of the same composition. Incubation was carried out at 34° and the flasks were carefully shaken twice daily in order to aerate the media. A positive test for malic acid was obtained on the fifth day, and increased in intensity up to the ninth day, whereupon the contents of all the flasks were combined, acidified with a little glacial acetic acid, and digested on the water-bath to coagulate the culture. The solution was filtered, further concentrated to 700 cc., treated with 140 g. of crystalline lead acetate in 200 cc. of water, and then made slightly alkaline with ammonia, after which 500 cc. of 95 % alcohol were added. Two days later the precipitate of lead salts was removed at the pump, washed with 30 % alcohol and dried. The material was triturated in a mortar and suspended in 100 cc. of water, and the lead removed by H_2S . On subsequent concentration to 75 cc. crystals of succinic acid separated on standing. These were removed and the mother liquor was then concentrated to 10 cc., when a further crop of succinic acid separated and was also removed. The residual syrupy liquid was neutralised with sodium hydroxide, diluted with water to 56 cc. and 4 cc. of glacial acetic acid were added. 25 cc. of this solution were mixed with 10 cc. of an 8 % solution of uranium acetate, and after standing in the dark showed a rotation of -2.4° (Ventzke scale). 25 cc. of a 1 % solution of authentic sodium *l*-malate plus 10 cc. of an 8 % uranium acetate solution showed, on similar treatment, a rotation of -13.7° . Assuming

the optical activity of the fermentation product to be due to *l*-malic acid, this result represents a yield of approximately 0.1 g. of the latter.

Exp. 2. This was a repetition of *Exp. 1*, with the exception that the fermentation was allowed to continue for a longer time. Malic acid was first detected on the 4th day. On the 14th day the bacteria were still active and a sample of the liquid gave a positive test for the presence of pyruvic acid when tested by Quastel's method. The whole of the culture medium was then worked up as in the previous experiment and the acid liquid was concentrated until succinic acid separated. The latter was removed and the process of concentration and removal of succinic acid was repeated until finally a viscous syrup, nearly free from succinic acid, was obtained. This was ground with anhydrous sodium sulphate and extracted with dry ether. The ethereal solution was concentrated to 20 cc. and treated with light petroleum until a faint turbidity appeared. On slow evaporation a brownish crystalline product was obtained. This sintered at 96° and a portion of it finally melted at 165°, hence it evidently still contained traces of succinic acid. The material was then shaken with 5 cc. of water and 10 cc. of ether. The aqueous extract, now entirely free from succinic acid, was almost neutralised with sodium hydroxide, evaporated to 5 cc. and heated on the water-bath with 0.8 g. of *p*-nitrobenzyl bromide in 10 cc. of alcohol. The product was recrystallised twice, when it had m.p. 114°. This value lies between that of the di-*p*-nitrobenzyl ester of *l*-malic acid (124–125°) and that of the di-*p*-nitrobenzyl ester of the *dl*-acid (109°). A mixture of the *dl*- and *l*-forms of malic acid in the ratio of 55 parts of the former to 45 parts of the latter was made from authentic materials and treated with *p*-nitrobenzyl bromide as above. The product after two recrystallisations had m.p. 115° and appeared identical with the esterified culture product.

Exp. 3. 3.5 litres of 2 % ammonium succinate medium were inoculated as before, but the fermentation was conducted at a lower temperature (30°). On the 13th day the malic acid test became strongly positive, whereupon the whole of the culture solution was concentrated to a syrup. Phosphoric acid was added and the mixture was treated with ether for several hours in a continuous-extraction apparatus. This afforded a light brown solid which was then treated with charcoal in hot aqueous solution. On evaporation of the filtered liquid there remained 27 g. of colourless crystals, m.p. 164°. These were redissolved in hot water and fractionally crystallised, when 7 g., m.p. 139–140°, were obtained. This was redissolved in water and extracted with ether. The aqueous layer on evaporation yielded 2.5 g. of slightly deliquescent crystals, m.p. 114–116°, and the ether extract gave material which, on further fractional crystallisation from hot water, afforded a small quantity of pure *dl*-malic acid, m.p. 130°. The substance of m.p. 114–116° gave tests for pure malic acid (found: equivalent, 66.4; calc. 67.0; found by micro-method: C, 35.2, 35.3; H, 4.4, 4.5; calc. C, 35.8; H, 4.5 %). A portion (0.111 g.) on polarisation in the presence of uranium acetate by Dakin's method [1924] gave a reading of – 6.3° (Ventzke scale) which indicated that 40 % of the material was *l*-malic acid. This was

confirmed by reference to a fusion-point curve of mixtures of *dl*- and *l*-malic acids which was constructed from determinations of the melting points of known mixtures. From this curve it was seen that a mixture of 60 % *dl*-acid with 40 % *l*-acid melts at 117°. A further small portion (0.1 g.) of the material of M.P. 114–116° was polarised in the presence of 10 % ammonium molybdate solution (Williamson's method [1918]), when a reading of + 2.1° (Ventzke scale) was observed, a final confirmation of the presence of *l*-malic acid. The remainder of the material was then fractionally crystallised from ether-light petroleum, when a very small amount of pure *dl*-malic acid, M.P. and mixed M.P. 130°, was finally separated.

*Development of dextrorotatory activity in cultures of
B. pyocyaneus on dl-malic acid.*

Pure *dl*-malic acid (60 g.) was dissolved in 2 litres of Ringer's solution and neutralised by addition of approximately 50 cc. of concentrated ammonia solution (sp. gr. 0.88). The solution was divided between eight flasks, sterilised, and adjusted to p_H 7.4 by addition of hydrochloric acid. After inoculation, followed by incubation at 34° for 7 days, the cultures were combined and evaporated to dryness. The residue was redissolved in a little water, acidified with phosphoric acid and extracted with ether as in Exp. 3 for several hours. The ether extract was dried and evaporated to a syrup which slowly crystallised in a desiccator. The material was tested for lactic acid by distillation with sulphuric acid, but the distillate showed no reaction for acetaldehyde, hence lactic acid was absent. Two quantities (0.578 g. and 0.711 g.) of the crystals were dissolved in 80 cc. of distilled water in each case. The solutions were clarified with charcoal, neutralised with potassium hydroxide, treated with a drop of glacial acetic acid and then each made up to 100 cc. A portion (10 cc.) of one of the solutions showed a rotation of + 5.4° (Ventzke scale) in the presence of uranyl acetate; a further 10 cc. showed a rotation of – 7.8° (Ventzke scale) in presence of ammonium molybdate. Hence Williamson's test [1918] for the presence of *d*-malic acid was positive. The specific rotations of the two solutions were then determined by Dakin's method, when an average value of 301.5° was found, corresponding to the presence of 62.5 % of *d*-malic acid in the crystals.

The whole experiment was repeated, the fermentation being allowed in this case to continue for 14 days.

Polarimetric examination of the product demonstrated the presence of 70 % of *d*-malic acid.

During the fermentations of *dl*-malic acid described above the presence of pyruvic acid was detected in the media by Quastel's test, the reaction being positive after 21 hours' incubation, and intense colorations were obtained after 45 hours. The concentration of this acid was too low, however, to permit of its isolation, only traces of precipitate being obtained with ketonic group reagents.

SUMMARY.

1. The results described in the present paper, when considered in conjunction with those recorded by Butterworth and Walker [1929], show that the growth of *B. pyocyaneus* on salts of citric acid results in precisely the same reactions as occur during the breakdown of this acid by the mould *A. niger* [cf. Challenger, Subramaniam and Walker, 1927].

2. The action of *B. pyocyaneus* on succinates also leads to the formation of the same products, *dl*-malic acid and *l*-malic acid, as are obtained by the action of *A. niger* on succinates. The evidence indicates that the *dl*-malic acid arises by direct hydroxylation of succinic acid and the *l*-malic acid by dehydrogenation of succinic acid to fumaric acid, followed by asymmetric addition of water to the latter.

3. From these observations in conjunction with other experimental data it is rendered likely that the formation of pyruvic acid from fumaric acid under the influence of *B. pyocyaneus*, as studied by Quastel, proceeds by the stages fumaric acid \rightarrow *l*-malic acid \rightarrow oxaloacetic acid \rightarrow pyruvic acid, and not in the more direct manner which experiments with methylene blue would appear to indicate.

4. A method, based on Denigès' mercuric acetate test, is described for the detection and approximate estimation of traces of malic acid in solution.

The authors' thanks are due to Dr J. H. Quastel for the expression of his opinion on certain points of interest arising out of this work.

REFERENCES.

- Aubel (1921). *Compt. Rend. Acad. Sci.* **173**, 179.
 ——— and Cambier (1922). *Compt. Rend. Acad. Sci.* **175**, 71.
 Brady and Elsmie (1926). *Analyst*, **51**, 77.
 Butterworth and Walker (1929). *Biochem. J.* **23**, 926.
 Challenger and Klein (1929). *J. Chem. Soc.* 1644.
 ——— Subramaniam and Walker (1927). *J. Chem. Soc.* **200**, 3044.
 Chodat and Pasmanik (1907). *Arch. Sci. Phys. Nat.* **23**, 386.
 Dakin (1924). *J. Biol. Chem.* **59**, 11.
 Denigès (1900). *Compt. Rend. Acad. Sci.* **130**, 34.
 Quastel (1924). *Biochem. J.* **18**, 365.
 ——— (1926). *Biochem. J.* **20**, 166.
 ——— Stephenson and Whetham (1925). *Biochem. J.* **19**, 304.
 ——— and Whetham (1924). *Biochem. J.* **18**, 519.
 Rimini (1904). *Z. anal. Chem.* **43**, 517.
 Stent, Subramaniam and Walker (1929). *J. Chem. Soc.* 1987.
 Thurlow (1925). *Biochem. J.* **19**, 175.
 Van Niel (1928). The propionic acid bacteria. (Uitgeverszaak Boissevain-Haarlem.)
 Williamson (1918). *J. Amer. Chem. Soc.* **40**, 693.

XVIII. THE ANTISCORBUTIC POTENCY OF APPLES. II.

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SOME recent publications [Bracewell, Hoyle and Zilva, 1930, 1, 2] have dealt with an exploratory investigation instituted with the object of ascertaining whether a functional relationship exists between the antiscorbutic activity and the physiological condition of the apple fruit. With this end in view the potency of a number of English and imported varieties of known environment was assessed and the influence of empirical methods of storage and of heat on the vitamin content of apples was studied. The following salient facts emerged.

(1) The Bramley's Seedling is markedly more potent than the other varieties tested, which definitely vary in activity among themselves but to a smaller extent.

(2) There is no indication, so far, that "age of tree," soil or season has any bearing on the antiscorbutic activity of the apple.

(3) There is little deterioration of the vitamin when apples are stored either at 1° in the air (cold store) or at 10° in an atmosphere of approximately 10 % oxygen, 10 % carbon dioxide and 80 % nitrogen ("gas" store); the loss is, however, greater in the case of the "gas" stored apples.

(4) Heating Bramley's Seedling apples in their skins at about 115° for approximately 50 minutes has little effect upon their antiscorbutic activity.

While these results offer no indication so far whether vitamin C has any physiological function in the plant, they nevertheless point to the fact that the following up of this enquiry is advisable, not only from the vitamin aspect but also from the botanical point of view. Without drawing any broad lines of demarcation, the investigation is now being continued in collaboration with groups of workers interested in different fields of pomological research. In view of the lengthy nature of the investigation and of the seasonal character of the experimental material, it is proposed, for the sake of convenience, to record the results at suitable intervals even if they do not yield information from which definite conclusions can be drawn at the time.

¹ Member of the Scientific Staff, Medical Research Council, working with grants for assistance and expenses.

The present communication deals with:

- (1) The antiscorbutic activity of further apple varieties.
- (2) The effect of low temperatures and of maturity on the antiscorbutic activity of the apple.
- (3) The effect of temperature of storage on the antiscorbutic activity of ageing apples.
- (4) The distribution of the vitamin in various parts of the apple.

The technique utilised in these tests was precisely the same as that already described [Bracewell, Hoyle and Zilva, 1930, 2].

The antiscorbutic activity of further apple varieties.

The varieties tested were Newton Wonder and Lane's Prince Albert.

The Newton Wonder apples were taken from fifteen year-old trees grown on a flinty loam over chalk, near Eynsford, Kent.

The Lane's Prince Albert apples came off 15-20 year-old trees grown on a chalk soil at Exning, near Newmarket.

The apples after being picked were stored at a constant temperature of 1° at the Low Temperature Research Station, Cambridge, and weekly consignments were delivered to London for testing purposes.

The test of the Newton Wonder apples began on 15th October, 1929, and that of the Lane's Prince Albert on 24th October, 1929.

Table I.

Dose g. daily	Lane's Prince Albert 1929	Newton Wonder 1929	Normal Bramley's Seedling 1929 Stored 3 rd 1st batch	King Edward 1929 Bristol High nitrogen	Worcester Pearmain 1928 Burwell	Normal Cox's Orange Pippin 1928 Burwell	Woodbine 1928 Bristol	Dabinett 1928 Bristol
3	+	+	+ + + +	No protection	No protection	+	No protection	No protection
5	+ + +	+ +	+ + + +	+	No protection	+	?	+ + +
10	+ + + +	+ + + +	+ + + +	+ + +	+	+ + +	+	+ + + +
20	+ + + +	Vitiated test	Test not finished, but fully protected for 60 days	+ + + +	+ + +	+ + + +	+ + + +	+ + + +

It will be seen from Table I, in which other varieties are incorporated for comparison, that the Newton Wonder showed an activity of the order of Cox's Orange Pippin whilst the Lane's Prince Albert, like the Dabinett, falls between the Bramley's Seedling and the Cox's Orange Pippin in vitamin activity. It will be of interest to ascertain whether further tests will confirm this result.

The effect of low temperature (-20°) and of maturity on the antiscorbutic activity of the Bramley's Seedling.

In the first investigation of the series it was found that the early gathering of Bramley's Seedling made no appreciable difference to its vitamin C content. It was next of interest to ascertain whether in the still earlier stages of the life cycle of the apple the concentration of the vitamin is of the same order as when the fruit is ready for gathering. If that were so, it would indicate that the vitamin is either synthesised by the apple or that it is supplied to it as it grows on the tree. Alternatively, it might have been supposed that even at this early stage the fruit contains the same amount of vitamin as when it is fully grown. In this event the antiscorbutic potency of the immature apple tissue would have been proportionately higher than that of the mature apple.

Bramley's Seedlings, about 3.2 cm. in diameter and 23 g. in weight, gathered at the end of July, and fully grown apples of similar origin gathered in the middle of October were tested for comparison. The fruit was picked from 20 year-old trees grown on flinty loam over chalk near Eynsford, Kent.

The mature and immature apples were placed immediately after gathering in a constant-temperature room kept at -20° at the Low Temperature Research Station at Cambridge, and weekly supplies were removed from the room and despatched to London in a well-insulated box. On arrival the fruit was transferred immediately to a refrigerator in which a temperature of approximately -12° was maintained. Quantities necessary for the test were removed daily, thawed and brought up to a temperature suitable for feeding to the experimental animals.

The test on the immature Bramley's Seedlings began on 8th August, 1929, that on the normal¹ apples on 21st October, 1929 (1st batch). The normal fruit was also kept in the hard frozen condition until 20th February, 1930 (2nd batch), when it was again tested for its antiscorbutic potency.

Table II.

Dose g.	Frozen immature Bramley's Seedling 1929	Frozen normal Bramley's Seedling 1929, 1st batch	Frozen normal Bramley's Seedling 1929, 2nd batch	Normal Bramley's Seedling 1929 Stored 3 ^o 1st batch
$\frac{1}{2}$	No protection	Not tested	Not tested	Not tested
1	+	"	"	"
3	+++	++	++	++++
5	++++	++++	++++	++++
7	++++	Not tested	Not tested	Not tested
10	++++	++++	++++	++++
20	Not tested	++++	++++	Test not finished, but fully protected for 60 days

The results are summarised in Table II. When compared with similar apples kept at 3^o the frozen apples show a slightly but definitely lower vitamin content. There are indications, however, that this small loss was not incurred

¹ The term "normal" is used throughout this paper to denote that the fruit was gathered at the normal time.

in the process of freezing but during the period which elapsed between the thawing of the fruit and its consumption by the experimental animals. Precisely the same titre was obtained with these apples after 4 month's storage.

The immature Bramley's Seedlings seem slightly more active than the normal apples. Had they, however, possessed the same total amount of vitamin as the fully grown apples, a daily dose of 0.5 g., or even less, of tissue would have been sufficient to protect a guinea-pig from scurvy. The amount of vitamin in the apple therefore increases as it grows on the tree.

The effect of temperature of storage on the antiscorbutic activity of ageing apples.

In view of the observations made that the loss in the antiscorbutic activity of apples is somewhat greater when the fruit is stored in an atmosphere of nitrogen, carbon dioxide and oxygen at 10° (*i.e.* "gas" storage) than when it ages in the air at 1°, it was thought desirable to ascertain whether this higher loss was brought about by the difference in the temperature or in the composition of the atmosphere of the two methods of storage. Bramley's Seedling apples gathered at the normal time, of the same origin as those which were employed in the preceding experiments, were therefore allowed to age in the air at 3° and at 10°. Unfortunately circumstances did not permit a comparison to be made with apples "gas"-stored at 10°. In the case of the Bramley's Seedling variety a displacement of the metabolic balance of the tissues occurs during storage at 1°, eventually leading to low-temperature breakdown, whereas at 3°, the metabolic activities of the fruit proceed normally; hence the choice of the latter temperature in this experiment.

The tests on the first batch of apples stored at 3° began on 21st October, 1929, that of the second on 6th January, 1930, and that of the third on 22nd April, 1930. The apples stored at 10° were tested on 2nd February, 1930.

Table III.

Dose g. daily	Normal Bramley's Seedling 1929 Stored 3° 1st batch	Normal Bramley's Seedling 1929 Stored 3° 2nd batch	Normal Bramley's Seedling 1929 Stored 3° 3rd batch	Normal Bramley's Seedling 1929 Stored 10°
3	+++	+++	+++	+++
5	+++	+++	+++	+++
10	+++	+++	+++	+++
20	Test not finished but fully protected for 60 days	+++	+++	+++

The results obtained in this experiment (Table III) yielded no definite answer as to whether the greater deterioration in the antiscorbutic activity of apples in "gas" storage was due to the temperature or to the composition of the atmosphere, since the slightly lower activity of the sample of apples stored at 10° for about 3 months as compared with that of the samples stored at 3° was of an order which falls within the limits of the experimental error of the

method employed in the assessment of vitamin C. It is of interest to note, however, that no loss of activity was recorded in Bramley's Seedling apples stored for 6 months at 3°.

The distribution of vitamin C in various parts of the apple.

In all the experiments of the preliminary and of this investigation the cortex of the peeled apples was used for the determination of the relative activity of the fruit. Experiments are here described in which the distribution of the vitamin in the fruit, carried out on some of the 1929 apples discussed in the preceding sections, is studied.

Three sections of the apples were tested, the peel, the outer half of the cortex and the inner half of the cortex of the peeled apples. The results are presented in Table IV. As it was not possible to use the usual number of 6 guinea-pigs, the number of animals employed in each test is noted in the table.

Table IV.

Normal Bramley's Seedling 1929. Stored 3°, 1st batch								
Dose g.	Peel	No. of guinea-pigs	Outer cortex	No. of guinea-pigs	Inner cortex	No. of guinea-pigs	Total cortex from peeled apples	No. of guinea-pigs
$\frac{1}{2}$	+++	4	Not tested	—	Not tested	—	Not tested	—
1	++++	4	—	—	—	—	—	—
$1\frac{1}{2}$	Not tested	—	"	3	"	—	"	—
3	++++	4	++++	2	++	3	++++	6
5	Not tested	—	++++	3	Vitiated test	—	++++	6
10	"	—	Not tested	—	"	—	++++	6
20	"	—	"	—	Not tested	—	Test not finished but fully protected for 60 days	6

Normal Bramley's Seedling 1929. High nitrogen sample						
Dose g.	Outer cortex	No. of guinea-pigs	Inner cortex	No. of guinea-pigs	Total cortex from peeled apples	No. of guinea-pigs
$\frac{1}{2}$	Not tested	—	Not tested	—	Not tested	—
1	"	—	"	—	"	—
$1\frac{1}{2}$	++	3	"	—	"	6
3	++++	3	++	3	++++	6
5	++++	3	+++	3	++++	6
10	Not tested	—	++++	3	++++	6
20	"	—	Not tested	—	Test not finished but fully protected for 55-60 days	6

Frozen immature Bramley's Seedling 1929				Newton Wonder 1929				
Dose g.	Peel	No. of guinea-pigs	Total cortex from peeled apples	No. of guinea-pigs	Peel	No. of guinea-pigs	Total cortex from peeled apples	No. of guinea-pigs
$\frac{1}{2}$	++	6	No protection	6	No protection	4	Not tested	—
1	++++	6	—	6	—	4	—	—
$1\frac{1}{2}$	Not tested	—	Not tested	—	Not tested	—	"	—
3	"	—	+++	6	++++	4	—	6
5	"	—	++++	6	++++	4	++	6
10	"	—	++++	6	Not tested	—	++++	6
20	"	—	Not tested	—	"	—	Not tested	—

The tests began at the following times.

Normal Bramley's Seedling apples, 1929, at 3°: peel October 2nd, 1929, outer cortex January 6th, 1930, inner cortex November 22nd, 1929.

Normal Bramley's Seedling apples, 1929. High nitrogen: outer cortex January 14th, 1930, inner cortex November 22nd, 1929.

Peel of frozen, immature Bramley's Seedling apples, 1929, on August 8th, 1929.

Peel of Newton Wonder apples, 1929, on 3 g. and 5 g. dose, October 15th, 1929, and on 0.5 g. and 1 g., November 13th, 1929.

It will be seen (Table IV) that in the case of the normal Bramley's Seedling apple in which the potency was determined in the peel, in the outer cortex and in the inner cortex, the peel is more than 3-4 times as active as the outer cortex, and over 6 times as active as the inner cortex and that the outer cortex, as might be expected, is somewhat more active than the total cortex of the apple. In the case of the immature Bramley's Seedling and of the Newton Wonder, the relationship of the activity of the peel to that of the total cortex of the apples is similar to that of the normal Bramley's Seedling.

In this connection it should be mentioned that Givens, McCluggage and Van Horne [1922] have found dried apple peel more active than dried apple. Scheunert [1930] also states that the vitamin activity of apples depends on the method of peeling.

It is impossible to say at this stage of the enquiry whether the disparity in activity between the different parts of the fruit has any physiological significance, but in view of the similar distribution of certain enzymes in the apple such a possibility must be borne in mind.

SUMMARY.

1. Newton Wonder apples possess a vitamin C content of the order of that possessed by Cox's Orange Pippin, whilst Lane's Prince Albert occupies an intermediate position between the latter and the much more potent Bramley's Seedling.

2. Bramley's Seedling apples can be frozen at -20° and stored at this temperature for 4 months without losing appreciably in antiscorbutic activity.

3. Bramley's Seedling apples gathered at the end of July are not more active per g. than the normal apple from the same plantation gathered in October.

4. Bramley's Seedling apples stored at 3° in air for 5 months do not lose any of their antiscorbutic activity.

5. The concentration of vitamin C in the tissue of the apple increases as the skin is approached from the core and is more than 6 times as great in the peel as in the flesh near the core.

REFERENCES.

- Bracewell, Hoyle and Zilva (1930, 1). *Biochem. J.* **24**, 82.
——— (1930, 2). *Med. Res. Council, Special Report Series*, No. 146.
Givens, McCluggage and Van Horne (1922). *Amer. J. Dis. Child.* **23**, 210.
Scheunert (1930). *Der Vitamingehalt der Deutschen Nahrungsmittel*. (Zweite Ergänzte Auflage. Julius Springer.)

XIX. THE ANTISCORBUTIC POTENCY OF APPLES. III.

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IN comparing the potency of a number of varieties of apples, a particularly striking disparity between the antiscorbutic activity of the Bramley's Seedling and the King Edward was observed [Bracewell, Hoyle and Zilva, 1930, 1, 2] in spite of the fact that these two apples resemble each other in so many respects. A few chemical analyses disclosed also a difference in their nitrogen content. Thus, three nitrogen determinations yielded the following percentages: Bramley's Seedling 0.027, 0.035 and 0.038, King Edward 0.061, 0.070 and 0.072. In view of these indications it became necessary to establish definitely whether a relationship existed between the nitrogen content and the antiscorbutic activity of the apple. In the experiment here described an attempt was made to throw some light on this subject. The aim was to investigate Bramley's Seedling and King Edward apples grown under conditions which would conduce to the production of fruits of high and low nitrogen content similar to the above in both varieties. The following are the cultural details of the experimental material.

King Edward VII.

High nitrogen sample. The fruits were grown at Long Ashton Research Station. The soil, derived from the Keuper Marl formation, is a medium loam containing high proportions of fine sand and silt. The trees were planted in 1920, are under clean cultivation and have received annual dressings of fertiliser as under:

Nitrate of soda	at 2 cwt. per acre
Superphosphate	„ 3 „
Sulphate of potash	„ 1 „

The trees are of the bush type, are extremely vigorous and growth is very strong. They are just beginning to come to the age of bearing crops.

The 1929 crop was very light and all the fruits were large and of the vigorous type. The fruits were picked on October 8th, 1929.

¹ Member of the Scientific Staff, Medical Research Council, working with grants for assistance and expenses.

Low nitrogen sample. These were grown at Bratton, Wilts. The soil is derived from the Chalk formation and is a typical, thin, chalky soil. The trees were planted in 1922 and are of the bush type. They have only made medium growth. Since 1927 they have been grown under a sward of clover which has reduced vigour and resulted in typical "low nitrogen" trees. Potash manuring has been the manurial treatment given.

In 1929 vigour of growth was low, the leaves were yellowish green, the fruits small, with pale yellow ground colour and usually a fair reddish flush denoting low nitrogen conditions. The crop was heavy and the fruits were picked on October 8th, 1929.

Bramley's Seedling.

High nitrogen sample. These fruits were grown at Faversham, Kent. The soil is of mixed origin, being derived from surface deposits overlying chalk, probably largely Brick Earth and Thanet Sands material. It has the texture of a medium loam, containing high amounts of fine sand and silt fractions. The trees are about 20 years old, large and of the bush type, and are extremely vigorous in growth. Cultivation has been on a high level ever since planting. Fertilisers containing nitrogen and phosphorus have been freely applied but potash manures have only been applied to any extent during the last few seasons.

In 1929, vigour of growth was strong and the fruits were large, green specimens, being typically those of high nitrogen trees. The crop was heavy. The fruits were picked on October 9th, 1929.

Low nitrogen sample. These were also grown at Faversham, Kent, in an orchard situated near that on which the high nitrogen sample fruits were grown. The soil is of similar origin to that of the high nitrogen sample but is heavier and the site is rather drier. The trees are of similar age and type to those from which the high nitrogen fruits were taken but have always been less vigorous, and the vigour has been greatly checked by substituting grass culture for arable conditions.

In 1929, the trees were showing all the symptoms of severe nitrogen starvation, *i.e.* lack of shoot growth, very sparse, small and yellow leaves and small fruits of extremely high colour. The crop was heavy. The fruits were picked on October 9th, 1929.

On analysis the following percentages of nitrogen were found: *King Edward*. High nitrogen 0.0390 and 0.0387; low nitrogen 0.0305 and 0.0309. *Bramley's Seedling*. High nitrogen 0.0404 and 0.0372; low nitrogen 0.0166 and 0.0172. The high and low nitrogen figures of both varieties did not, unfortunately, coincide with the corresponding high nitrogen figures of *King Edward* and the low nitrogen figures of the *Bramley's Seedling* previously tested. In fact, the high nitrogen *King Edward* in this experiment contains about 30 % or 40 % less nitrogen than the apples of this variety used in the first experiment, whilst the nitrogen content of the high nitrogen *Bramley's Seedling* is

of the same low order shown by these apples in that investigation. There is, however, a definite difference in the nitrogen content in both cases.

The difference in the antiscorbutic activity between the high and low nitrogen Bramley's Seedling (Table I) falls within the limits of the experimental error of the method of the determination of vitamin C. The low nitrogen King Edwards are, on the other hand, definitely (about 1.5 times) more potent than the high nitrogen apples.

Table I.

These tests began on October 27th, 1929.

Dose g. daily	Bramley's Seedling 1929 Low nitrogen	Bramley's Seedling 1929 High nitrogen	King Edward 1929 Low nitrogen	King Edward 1929 High nitrogen
3	+	+	+	+
5	+	+	+	+
10	+	+	+	+
20	Test not finished but fully pro- tected for 55-60 days	Test not finished but fully pro- tected for 55-60 days	+	+

These results, however, offer no clear-cut information for or against the theory that the nitrogen content of the apple has a bearing on its vitamin C content and consequently further attempts are being made in the hope of ascertaining whether such a relationship exists.

SUMMARY.

King Edwards containing about 0.0307 % of nitrogen were about 1.5 times as potent antiscorbutically as apples of this variety containing about 0.0387 % of nitrogen. No significant disparity in the vitamin C content was found between Bramley's Seedlings containing high and low quantities of nitrogen.

REFERENCES.

- Bracewell, Hoyle and Zilva (1930, 1). *Biochem. J.* **24**, 82.
 ——— ——— (1930, 2). *Med. Res. Council. Special Report Series*, No. 146.

XX. THE BIOLOGICAL OXIDATION OF CARBOHYDRATE SOLUTIONS.

PART I. THE OXIDATION OF SUCROSE AND AMMONIA IN SECTIONAL PERCOLATING FILTERS.

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THE manufacture of sugar from the sugar beet gives rise to large volumes of waste water of a polluting nature. About five-sixths of the beet sugar factory effluent originates from the water which is used to wash and convey the beet from open storage silos or from trucks into the factory. Under usual conditions the conveying or fluming water requires only simple treatment so that it may be used again in the factory. The remaining one-sixth of the total effluent is obtained from the beet pulp, which after extraction of sugar by diffusion, is dried and used as cattle food. Before the spent pulp is dried, as much water as possible is squeezed out of it. A "press water" is thus obtained which is highly polluting by reason of dissolved organic substances. Numerous attempts have been made on the Continent to devise a process which would destroy the dissolved substances but the methods tried have not been attended with great success.

Press water is a liquid of varying composition, but to illustrate its nature the following analysis of a sample taken from a factory is given; sucrose 230, protein 23, pectin 4.3, pentosan 13.4 and saponin 0.2 parts per 100,000. In view of the pollution already experienced and the increase that would probably follow as a result of the extension of the beet sugar industry, investigations were begun in 1927 at the Rothamsted Experimental Station and at the Colwick factory of the Anglo-Scottish Beet Sugar Corporation, Nottingham. for the Department of Scientific and Industrial Research with the object of ascertaining whether liquids such as press water could be satisfactorily purified by biological oxidation on percolating filters. The results of the semi-commercial scale experiments at Colwick have already been briefly described in the first three annual reports of the Water Pollution Research Board of the Department of Scientific and Industrial Research [1928, 1929, 1930]. At Rothamsted preliminary experiments showed that effective oxidation of solutions of the approximate composition of press water could be achieved, after dilution, by filtration through biological or percolating filters. The filters consisted of earthenware drainpipes 24 inches deep and 6 inches diameter filled with 1/8 to 1/4 inch clinker. In order to obtain artificial press water, sliced mangolds

or sugar beet were first boiled with water, the sugar extract strained off, and the residual pulp was then squeezed in a press. The runnings were diluted to the desired strength. Such artificial press liquors were most effectively oxidised when diluted so as to contain 0.1 % of sucrose, and at this strength they were allowed to trickle continuously on to the filters at a rate of 4.6 cc. per minute, equivalent to 100 gallons per cubic yard of filter per day. In this manner 90-95 % of the sugar was oxidised.

It was observed that the mangold or beet extracts rapidly became acid, owing to the fermentation of the sugar. Thus the p_H value of the freshly made solutions fell from 7.5-8.0 to 3.0-5.0 in less than 24 hours. The effluent obtained from the filtration of such an acid solution, however, was invariably neutral or slightly alkaline. When the acid fermented solutions were made alkaline with milk of lime and then filtered, the activity of the micro-flora seemed to be impaired in some way and an effluent of inferior quality resulted. In later experiments it was found that when a solution made from beet press water was fermented for 8 hours at 35°, the products were approximately 45 % unchanged sugar, 40 % hydroxy-acids and 15 % aliphatic acids. The behaviour on filtration of solutions composed of sugar and such typical products of fermentation as acetic and lactic acid was therefore studied in some detail. The results of these experiments proved conclusively that the course taken by natural fermentation provides a suitable liquid for oxidation on percolating filters; a mixture of sugar, acetic and lactic acids derived from a fermented solution of sugar is more easily oxidised than the original sugar solution. Only a partial conversion of sugar to acid is desirable. It seems that the acids alone do not provide the filter organisms with the most suitable food material to encourage the growth of a highly efficient biological film; the acids are then only partially oxidised. Moreover, filters fed with either acetic or lactic acid require added nitrogen, whereas those provided with a mixed diet of sugar and acid appear able to utilise very small amounts of nitrogen or possibly to fix atmospheric nitrogen. An interesting observation made by Dr Sandon, of the Rothamsted Experimental Station, was that all the filters, whether fed with sugar, sugar plus acetic acid, sugar plus lactic acid, lactic acid, acetic acid or any similar combination with different forms of added nitrogen, seemed to support the same protozoan and insect population.

One of the most significant observations made during the 1928 experiments was the presence of nitrates in incompletely oxidised effluents. These experiments were carried out with the following solutions: (a) 0.1 % sugar with 0.003 % of nitrogen added as ammonium chloride, and made up in tap-water containing 0.0005 % of nitrogen as nitrate; (b) as (a) but with 0.003 % of nitrogen added as albumin. The effluents regularly contained 0.0015 % of nitrogen as nitrate when as much as 0.01 % of sugar was present at the same time. There was thus some evidence that nitrification may occur in presence of organic matter. This conflicts with the generally accepted view in sewage purification that oxidised nitrogen does not appear in an effluent until the

organic matter has been decomposed. Oxidation of carbon precedes that of nitrogen when solutions of organic nitrogenous compounds are stored in bottles and supplied with an ample quantity of oxygen. With asparagine, for instance, Adeney [1908] found that hydrolysis first takes place, giving aspartic acid and ammonia. This is followed by complete oxidation of the aspartic acid before the ammonia is nitrified. Although the results of previous investigations on the oxidation of dissolved substances have been confirmed, the generally accepted view that easily oxidisable carbonaceous matter inhibits nitrification on percolating filters was not supported by the results obtained with drainpipe filters. The frequent presence of nitrate and sugar in the laboratory filters might conceivably be explained by assuming that some of the sugar in the fresh liquor always side-tracked down the filter or ran straight through the medium and that such solution would form one of the numerous integral parts of the whole effluent. This possibility is dealt with in the present paper.

In some earlier experiments on biological filtration in 1927, mangold extracts containing 0.1 % of sucrose were run through drainpipe filters 2 feet deep. The disappearance of organic matter was measured at depths of 6, 12, 18 and 24 inches by means of the oxygen taken up by the sample from acid permanganate in 4 hours, with the result that the upper half of the filter removed the greater part of the organic matter just as in sewage filters. It is apparent that where the food supply is richest and the air supply is plentiful, micro-organisms will become more abundant. They are then able to utilise the strong solutions which are run on to them. Moreover, with liquids such as sewage, the bacteria and zoogaea can coagulate colloidal or suspended organic matter and then live on the coagulum. The lower part of a biological filter is thus apparently inefficient and contains less vital film only because its food supply is limited by the activity of the upper part.

The work described in the present paper was undertaken with the following objects:

- (1) to account for the advantage of oxidising a solution of sugar partly fermented to acids over that of an unchanged solution of sugar;
- (2) to follow the course of sugar decomposition and nitrification of ammonia through different levels of a percolating filter;
- (3) to ascertain the function of different sections of a percolating filter.

EXPERIMENTAL.

Apparatus and methods of analysis.

Apparatus (Fig. 1). The sectional filter used in these experiments consisted of 6 glass cylinders each 6 inches in diameter and 9 inches deep. The cylinders rested in perforated zinc trays supported on the ledges of a vertical frame. Liquid passing through the perforations was caught on a funnel and then dropped on to the next section. In order to obtain a constant flow of sugar

solution through a tube wide enough to be cleaned easily of bacterial growths, the apparatus shown in Fig. 1 was used. This device controls the hydrostatic head of solution in an aspirator by means of a length of fine capillary tubing. The sections were filled with $1/8$ to $1/4$ inch clinker which had been well washed after use in similar filtration experiments.

Methods of analysis. Sucrose. (a) This was determined by the Hagedorn-Jensen method as modified by Hanes [1929]: 2.5 cc. of the solution were inverted with 1 cc. NH_2SO_4 by heating on a boiling water-bath for 20 minutes and then neutralised with 1 cc. of N NaOH . The sucrose was returned as glucose. The limits of the Hanes modification are from 1.74 to 4340 mg. glucose per 100 cc.

(b) An alternative modification has been used for the range of 0.347 mg. to 3470 mg. glucose per 100 cc. A solution is made up containing 3.3 g. potassium ferricyanide and 10.6 g. sodium carbonate per litre. 10 cc. of this solution and from 0.1 to 10 cc. of the unknown solution are heated according to the usual procedure and the residual ferricyanide is finally determined by reducing it with KI and titrating with $\text{N}/200 \text{ Na}_2\text{S}_2\text{O}_3$.

Invert sugar. The Hagedorn-Jensen solutions were used but 10 cc. of the ferricyanide solution were taken instead of 2 cc. and the volume of invert sugar made up to 10 cc. The range of the method is thus greatly increased, the difference between the blanks on successive days is also reduced and more reliable results are obtained than in the original Hagedorn-Jensen method. While the limits of the latter method are from 0.868 mg. to 347 mg. glucose per 100 cc. those of the modified method are from 0.174 mg. to 1735 mg. per 100 cc.

Nitrogen as NH_3 . (a) Whenever the volume of solution permitted 250 cc. were distilled with ammonia- and nitrogen-free NaOH made according to the Ministry of Health Methods of Analysis [1929, p. 20]. The distillate was titrated with $\text{N}/25 \text{ H}_2\text{SO}_4$ using cochineal as indicator.

(b) When the above method was impracticable 50 cc. of the solution were evaporated to dryness in a water-bath and the residue was taken up in 5 cc. N NaOH and 50 cc. 95 % alcohol. This solution was then steam-distilled in Foreman's apparatus [1928] until 100 cc. of distillate were collected and the ammonia was titrated with $\text{N}/25 \text{ H}_2\text{SO}_4$. It was found that this method gave a complete recovery of ammonia from dilute solutions of ammonium salts.

Nitrogen as NO_2 . This was determined colorimetrically as described in the Ministry of Health Methods [1929, p. 8].

Nitrogen as NO_3 . The residue in the steam distillation flask left after the determination of ammonia by Foreman's method was neutralised with normal acid and made up to 200 cc. One-half of this solution was reduced by incu-

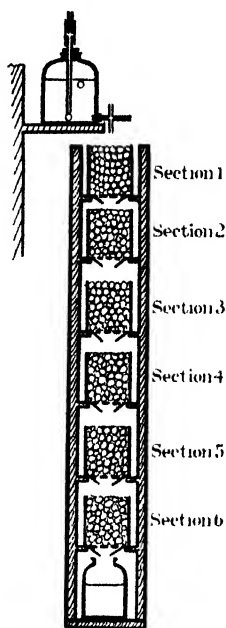


Fig. 1. Sectional percolating filter.

bation overnight at 35° with a zinc-copper couple; the ammonia was then distilled off and estimated by nesslerisation. The remaining half of the solution was incubated without the addition of a reducing agent, distilled and the distillate nesslerised. This represents the blank on the method. Details of the method are given in the Ministry of Health Methods [1929, p. 11].

Oxygen absorbed from permanganate in 4 hours. 10 cc. of $N/8$ $KMnO_4$ and 10 cc. of 25 % H_2SO_4 were incubated for 4 hours at 35° with sufficient solution of sugar or effluent to reduce not more than one-half of the permanganate. The unchanged permanganate was estimated with $N/20$ sodium thiosulphate. The amount of permanganate reduced is practically proportional to the sugar in the solution, provided the quantity of permanganate decomposed remains constant.

Biological oxygen demand, i.e. the oxygen absorbed from solution in 5 days or 20 days. In order to carry out this determination the dissolved oxygen present in a mixture of tap-water and the solution under examination is found. The mixture is then incubated for either 5 or 20 days at 20° and the oxygen absorbed from solution in the oxidation of the organic matter is obtained from a second oxygen determination. Under rigidly controlled conditions the oxygen taken up is approximately proportional to the organic matter present in the diluted solution.

p_H value. This was determined with the Hellige apparatus. The figures obtained gave good agreement when checked by the quinhydrone electrode method.

Total nitrogen. Before the solution was analysed as in the Kjeldahl method the nitrates and nitrites present were first reduced in the way described under *Nitrogen as NO₃*.

EXPERIMENTS.

Exp. 1. Filtration of a mixture of sucrose, and acetic and lactic acids. A solution composed of 0.033 % sucrose, 0.033 % acetic acid and 0.033 % lactic acid was made up in Harpenden tap-water and run on to the sectional filter at a rate of 9.0–9.5 cc. per minute. For the first 42 days the removal of organic matter from solution was measured only by the oxygen absorbed from permanganate and the biological oxygen demand tests. Adequate information on the rate of maturation of these filters had already been obtained and this information will shortly be published; hence no detailed analyses were made in the early stages of the experiment. The experiment proved that the acids were rapidly oxidised in the first and second sections. Thus the p_H of the original mixture which was 4.65 increased to 6.0 in the first section and 6.5 in the second; it then increased uniformly until the liquid was discharged from the sixth section at a p_H of 7.5. It should be noted that the nitric nitrogen in the final effluent during this period never exceeded 0.2 part per 100,000, although in the tap-water used to make up the nutrient solution there was 0.5 part of nitric nitrogen present.

Exp. 2. Filtration of a solution of sucrose and ammonium chloride. The further filtration of sucrose, and acetic and lactic acids was postponed on the 43rd day until suitable methods for the exact analysis of the substances in dilute solutions were available. From that day until the 88th day the following solution was fed to the filter at a rate of 9.5 cc. per minute.

Sucrose	1.0 g.
NH ₄ Cl	0.1528 g. (increased on 120th day
K ₂ HPO ₄	0.002 g. to 0.191 g.)
MgSO ₄ ·7H ₂ O	0.0006 g.
NaCl	0.0002 g.
CaCl ₂ ·6H ₂ O	0.1375 g.
Fe ₂ Cl ₆	Trace
Distilled water	1 litre

C/N ratio until 120th day = 10/1: thereafter 8/1.

Analyses were made every few days over a period of 45 days and the results obtained are given in Table I.

Table I. *Percentage of sugar oxidised.*

Section	...	1	2	3	4	5	6
By 4 hours' test		22.5	39.0	46.0	53.2	58.4	67.7
By 5 days' test		25.2	42.6	44.4	50.2	56.5	65.5

The nutrient solution was made up daily and adjusted to a p_H value of 7.0.

As the filter began to mature a drop in p_H of the effluent from the first section indicated that acid was being produced in this part of the filter. For example, on the 58th day of the experiment the p_H figures were:

	Section					
Fresh liquid	1	2	3	4	5	6
7.0	6.6	6.9	7.3	7.3	7.4	7.4

Similar observations have previously been made in the Microbiology Department at Rothamsted.

The p_H figures given were obtained during about the first half hour, at the commencement of each day's run. Thereafter the p_H of the fresh liquid fell progressively during its 24 hours' storage. Even by careful sterilisation of the aspirator it was difficult to prevent the p_H of the nutrient solution from falling below 5.5 to 6.0 at the end of the day. As a rule, however, the aspirator was cleaned out daily with distilled water and every few days with hydrochloric acid. Under these conditions a liquid of p_H 6.5 or less was filtered for the greater part of the day and there was no measurable production of acid. Typical examples are given in Table II.

Table II. *p_H of nutrient solution and sectional effluent.*

	Section					
Sucrose solution	1	2	3	4	5	6
6.5	6.8	7.3	7.4	7.4	7.4	7.4
5.5	6.6	6.9	7.0	7.1	7.2	7.2
4.6	6.4	6.8	7.0	7.1	7.2	7.2

Nitrogen assimilation. The nitrogen supplied as ammonium chloride was assimilated by the micro-organisms present in all the sections, but by those in No. 1 more than any other. It was evident from inspection that No. 1 section contained the bulkiest film. Compared with the other sections it oxidised the largest proportion of sugar. This is clearly shown in Table III.

Table III. *Nitrogen present in nutrient solution and effluents.*

Day of experiment	Nutrient solution	Section					
		1	2	3	4	5	6
54th	4 000	3.752	2.960	2.738	—	—	1.949
73rd	4 000	2.744	2.632	2.464	2.464	2.072	1.624
86th	5.000	4.704	3.472	—	3.248	—	2.688
112th	4 000	3.892	3.612	3.416	3.258	2.940	2.310
122nd	5.000	4.021	3.789	3.428	3.092	2.890	2.576
128th	5.000	4.372	4.230	—	—	—	—

It is apparent that nitrogen is continuously removed from the solution. This does not always signify an increase in vital film and therefore a correspondingly greater percentage oxidation of sugar; nitrogen may be lost by the decay of the organic film and its discharge as humus or by the removal of living micro-organisms in the final effluent or by the breeding and growth of flies on the filter, as rapidly as it is assimilated as ammonia.

Nitrification. In proportion to the amount of nitrogen locked up by the filter the quantity of nitrogen oxidised to nitrous and nitric acid is quite considerable. There appears to be a fine balance between the amount of nitrogen assimilated and carbohydrate oxidised on the one hand, and the amount of nitrogen oxidised. Where the film is thick and air passage somewhat restricted as in the upper sections, carbohydrate combustion is vigorous and nitrogen assimilation is appreciable. But in the lower sections, where organic matter is present in low concentrations (from 0.01 to 0.05 % sucrose) the filter is specialised for nitrification. It will be seen from Table IV, however, that

Table IV. *Nitrification in presence of ammonia and sucrose.*

Section	86th day Rate 9 cc. per min.				99th day Rate 5 cc. per min.				112th day Rate 5.85 cc. per min.			
	% sugar oxidised	N present as			% sugar oxidised	N present as			% sugar oxidised	N present as		
		NH ₃	NO ₂	NO ₃		NH ₃	NO ₂	NO ₃		NH ₃	NO ₂	NO ₃
1	9.4	4.66	0.01	0.03	10.8	5.0	0.01	0.0	37.6	3.89	0.05	0.17
2	9.7	3.43	0.01	0.03	24.0	4.3	0.01	0.12	4.4	3.61	0.05	0.20
3	6.7	—	0.01	0.03	5.3	4.2	0.02	0.24	6.3	3.42	0.05	0.29
4	8.7	3.17	0.01	0.07	28.6	3.0	—	—	9.9	3.26	0.06	0.32
5	10.7	2.08	0.01	0.15	—	2.5	0.03	0.25	8.3	2.94	0.07	0.37
6	22.0	2.57	0.02	0.10	6.6	—	—	0.52	11.6	2.31	0.05	0.61
Total	66.2				75.3				78.1			

complete absence of carbohydrate is not essential before nitrification can occur. On the 112th day, for instance, the effluent from Section No. 4 contained 44 parts of sucrose per 100,000, 0.06 part of nitrogen as nitrite and

0.32 part of nitrogen as nitrate. The solution passing through the filter receives a thorough mixing and it is therefore highly improbable that a small volume of nutrient solution passes through four sections and escapes oxidation while the rest of the solution undergoes a more complete oxidation. In other words, bacteria capable of oxidising ammonia to nitrite and nitrite to nitrate are actively present in a solution which contains free ammonia and carbohydrate.

Exp. 3. Interchange of sections Nos. 1 and 2. The effect of interchanging section No. 1 with No. 2 was studied from the 129th day of the experiment to the 157th. While section No. 2 showed a rapid increase in efficiency, due to the fact that it received a stronger nutrient solution, No. 1 fell off slowly. The net effect of the change, therefore, is to increase the capacity of the two upper sections to oxidise sugar. This is shown in Table V.

Table V. *Percentage of sugar oxidised.*

Section	Percentage of sugar oxidised.						
	Day before change	1st day of change	3rd day of change	6th day of change	14th day of change	20th day of change	29th day of change
No. 1	32.1	10.7	24.2	22.9	26.9	32.2	40.3
No. 2	8.0	34.4	35.9	24.4	21.4	17.0	26.8
No. 1 + No. 2	40.1	45.1	60.1	47.3	48.3	49.2	67.1
All sections 1 to 6	78 (about)	—	—	—	89.1	—	100.0

It was shown in Exp. 2 that the production of acid in Section No. 1 depends on the p_H of the nutrient solution. If this is less than 6.7 or 6.8 then increase in acidity cannot be detected. The interchange of Sections Nos. 1 and 2 gave additional evidence in support of the view that the p_H of the nutrient solution controls acid formation. Thus in Table VI it is seen that for 2 days after Section No. 1 had been placed in No. 2 position, it received a liquid of p_H 6.8. This section still retained its powers of acid production, for the p_H was lowered to 6.6. A few days after the change, however, it was unable to produce acid from a liquid of p_H 6.8.

Table VI also indicates the rapid oxidation of acid, even by an immature section such as No. 2.

Table VI. *Effect of interchange on p_H values.*

	Day before change	1st day of change	2nd day of change	3rd day of change	6th day of change	14th day of change	29th day of change
Nutrient solution	6.0	6.3	6.8	6.0	4.4	6.0	6.6
Section No. 1	6.4	6.8	6.8	6.6	6.0	6.8	6.6
Section No. 2	6.4	6.6	6.6	6.8	6.4	6.9	6.8

Estimations of nitrate in the effluents of the sections interchanged were made at frequent intervals. These analyses are given in Table VII. It may be stated that the average amount of nitrate present in the effluent from the first section did not exceed 0.05 part N per 100,000, while in the second section this increased to 0.1 part. When the change of section was made nitrate was

definitely absent from the first effluent after the 6th day. The effluent from the next section (No. 1 temporarily in the place of No. 2) contained appreciable amounts of nitrate, though the results were somewhat erratic. However, sufficient nitrate was present to prove that nitrification had actually occurred in the presence of sucrose and invert sugar, equivalent in concentration to 0.06 to 0.07 % of glucose.

Table VII. *Nitrogen as nitrate.*

		Parts per 100,000.				
Day before change	Section	1st day of change	3rd day of change	6th day of change	14th day of change	29th day of change
Section No. 1 0.105	No. 2	0.095	0.056	0.024	0.0	0.0
Section No. 2 0.114	No. 1	0.125	0.111	0.048	0.378	0.200

Exp. 4. Interchange of Sections Nos. 1 and 6. In order to confirm the results obtained in Exp. 3, Section No. 6 was made the first section and No. 1 the last. This experiment brings out the effect of a 0.1 % sucrose solution on the biological activity of nitrifying organisms. It also demonstrates the reaction of a mixed biological flora to solutions of ammonia and nitrite in which little carbohydrate is present.

Turning to the consideration of the course of the sugar oxidation, it is seen that the oxidising power of Section No. 6 rapidly increased in its new position. Prior to the interchange the progressive removal of sucrose as the solution percolated through the filter was of the following order: Section No. 1, 40.3 %; No. 2, 26.8 %; No. 3, 6.8 %; No. 4, 11.8 %; No. 5, 12.0 %; No. 6, 2.3 %; total 100 %. When these figures are compared with those given in Table VIII, the development of active film in Section No. 6 is shown by the greater proportion of sugar removed by this section. Within one month it was as active as either Sections 1 or 2, when these received the fresh solution first.

Table VIII. *Percentage of sucrose oxidised.*

Day before change	Section	1st day of change	4th day of change	7th day of change	11th day of change	27th day of change
Section No. 1 40.3	No. 6 (now No. 1)	14.9	18.4	21.5	14.5	42.5
Section No. 6 2.3	No. 1 (now No. 6)	-	---	-	15.8	12.5

It is a remarkable fact that the filter, considered as a whole, did not suffer by the sudden change of conditions in this experiment. This is due partly to the fairly slow reduction in rate of oxidation shown by Section No. 1, partly to the efficiency of this latter section in the last position even after one month and partly to the increasing activity of Section No. 6 in the first position. That the overall efficiency of the filter was not impaired is seen from the following example of the percentage removal of sugar effected by the sections in the order they were present in the filter: Section No. 6, 42.5 %; No. 2, 13.9 %; No. 3, 14.9 %; No. 4, 9.0 %; No. 5, 1.7 %; No. 1, 12.5 %; total 94.5 %.

For the first few hours of the change of Sections Nos. 6 and 1, nitrite and nitrate were found to be present to the extent of 0.018 and 0.190 part N per 100,000 respectively in No. 6 effluent (temporarily No. 1); in No. 1 effluent (temporarily No. 6), the amounts were 0.036 and 0.230 respectively. Within one week nitrifying activity had almost ceased in No. 6, only 0.008 part of nitrogen as nitrite and 0.04 part as nitrate per 100,000 being obtained after 6 days. Section No. 1, however, became more active in this respect: the effluent contained 0.04 part of nitrogen as nitrite and 0.44 part as nitrate. There seemed to be a tendency for nitrate to appear suddenly in Section No. 6 and then disappear. Insufficient evidence exists on which to base any satisfactory explanation of this apparent anomaly.

Inversion of sucrose during filtration. When sucrose solutions were run on to non-sectional percolating filters only small amounts of invert sugar were found in the effluents. Analysis of samples taken at various levels of the sectional filter used in the previous experiments proved that a considerable fraction of the total sucrose was invariably changed into invert sugar. Typical results are given in Table IX.

Table IX. *Sucrose and invert sugar.*

Sample	Calculated as mg. glucose per 100 cc.							
	102nd day of experiment		122nd day of experiment		157th day of experiment		185th day of experiment	
	Sucrose	Invert sugar	Sucrose	Invert sugar	Sucrose	Invert sugar	Sucrose	Invert sugar
Fresh solution	108.02	1.10	112.34	0.86	130.9	—	111.50	—
Eff. No. 1	81.57	8.67	73.39	2.25	67.35	10.85	53.54	10.54
Eff. No. 2	68.04	9.88	56.88	4.83	33.28	9.72	37.90	10.75
Eff. No. 3	55.23	11.97	48.23	6.97	28.82	5.38	22.31	9.68
Eff. No. 4	37.66	15.26	36.15	11.15	10.44	8.26	15.26	6.73
Eff. No. 5	33.02	12.85	34.63	11.37	0.00	3.05	14.47	5.66
Eff. No. 6	35.25	11.75	32.11	11.69	0.00	—	4.07	2.34

It is seen from Table IX that appreciable amounts of invert sugar are present in certain sections of the filter. The quantity of invert sugar produced by any particular section does not remain even approximately constant during the development of the biological film. It is not improbable that the limiting factor in the oxidation of sucrose is the rate of oxidation of the invert sugar. Hence, when the filter was still immature (up to about the 130th day) invert sugar would tend to accumulate in the lower sections, owing to the inability of the organisms in these sections to decompose as much invert sugar as they produced. After the 130th day the filter reached maturity. The lower sections were then able to oxidise invert sugar *in situ* as rapidly as it was formed, in addition to part of that presented by the upper sections.

DISCUSSION.

The experiments described in this paper prove that a biological film may be built up on a filter made up of sterile clinker by allowing a 0.1 % solution of sucrose and an adequate supply of available nitrogen to percolate slowly

through the filter. If the rate of flow of this nutrient solution is kept low and aerobic conditions are maintained, any desired degree of oxidation can be effected. Before the microbial population has become established the clinker removes a considerable proportion of the sugar by adsorption but once the organisms begin to develop loss of sugar due to purely physical forces becomes insignificant. The sugar is then oxidised entirely by the micro-organisms. When the part played by each section of the filter used for these experiments is examined, it should be remembered that the first section is given an advantage in the initial stages which becomes more pronounced as the filter matures. This advantage lies in the concentration of sugar in the nutrient solution which is run on to No. 1 section. Table 1 exhibits the beginning of the superiority of Section No. 1 over all other sections. As this section oxidised 25 % of the sugar, the second section can only decompose 18 % of the total sugar, assuming an efficiency equal to that of the first section. Each development of film in the top section therefore increases the difference between the amount of sugar oxidised by Section No. 1 and by lower sections. Further advantages which the top section gains are due to the fact that the water-holding capacity of the film and its absorptive power increase with the growth of film. The sugar therefore remains in contact with micro-organisms for a longer period in this part of the filter than in any other. A quantitative expression of the average time of contact of liquids with filter medium has already been worked out by an elaboration of Clifford's method [1907] and it has been shown that the time of contact might increase to two or three times the contact of the clean, sterile medium, as a result of film development. The sectional filter was not regarded as mature until it was able to oxidise 90 to 100 % of the sugar it received. The percentage of sugar oxidised by the different sections was then approximately: No. 1, 40 %; No. 2, 25 %; No. 3, 12 %; No. 4, 10 %; No. 5, 7 %; No. 6, 6 %.

Under suitable conditions each part of a filter is able to perform the work of any other section merely by interchanging the sections. An example of the operation of this principle is shown in Exp. 4. In this case the sixth section, which contributed very little towards the total oxidising effect of the whole filter, but was highly specialised for nitrification, was made the first section for one month. During this period microbial counts showed that there was a rapid development of yeasts, bacteria and protozoa in this section which corresponds with a parallel increase in the amount of sugar oxidised. At the end of the month as much sugar was completely oxidised to CO_2 and water by the sixth section (temporarily the first) as by the section previously in the first position. Even after one month, however, the latter section was able to decompose more sugar than the original sixth section and at the same time effect a considerable nitrification. It thus appears that a filter gains in efficiency when its various parts are matured by receiving in turn the strong nutrient solution.

The sequence of changes which the sugar undergoes in its ultimate

breakdown to CO_2 and H_2O is so far mainly a matter for speculation. A mixed flora exists on the filter and contains organisms which may bring about almost every known type of fermentation with the appropriate end products. In a mature aerobic filter the population co-operates in oxidising the sugar and only such organisms will exist in any quantity as can effectively make use of the sugar and of the waste products of other organisms. Intermediary products will thus tend to be oxidised as soon as they are formed. There is an alternative possibility that the usual products of fermentation are not formed to any extent under aerobic conditions of a percolating filter, or if formed are capable of rapid oxidation *in situ* by the organism which produces them. This idea gains in probability when it is remembered that typical products of bacterial fermentation are relatively stable in anaerobic cultures but break down to CO_2 and water when air is admitted to the cultures. Lactic acid, for example, accumulates in anaerobic yeast fermentations but disappears when the yeast is aerated [Fürth and Lieben 1922], or when the fermented liquid is treated on a percolating filter (unpublished work). There is little doubt, however, that a considerable proportion of the sucrose is first inverted as an intermediate stage in the oxidation. There is some evidence that an acid product is also formed, but the nature of this substance is as yet unknown. It has already been stated that the drop in p_{H} —evidence on which this claim is based—is recorded as the nutrient solution passes out of the first section. The latter is thick with film, which might restrict the passage of air and so produce partially anaerobic conditions and acid fermentation in those parts of the film not exposed to air.

Turning to a consideration of the role of nitrogen in the sectional filter, it is apparent that a constant supply of available nitrogen is necessary for the development and maintenance of the biological film; the greater the amount of active film the greater the demand for nitrogen. It has been shown in large-scale filters (unpublished work) that for reasons already stated the quantity of film steadily decreases from the top of the filter to the bottom. The results of the work described in this paper also show that there is a gradient of nitrogen assimilation, or protein synthesis, corresponding to the amount of film present in different levels of the filter. When an excess of nitrogen over and above this amount is added, nitrites and nitrates are produced. These compounds appear in effluents from the middle and lower sections of the filter even in the presence of appreciable quantities of sugar. For example, pronounced nitrification has occurred over long periods in solutions containing up to 60 parts of sucrose per 100,000. Similar observations were made two years ago when, as previously stated, the filter was not divided into sections. Since the work of Winogradsky [1891], it has been held that small concentrations of organic substances inhibit or prevent nitrification. This belief has repeatedly been attacked, as, for instance, by Beijerinck [1914]. There is, in fact, much conflicting evidence in support of either view. Until recently no satisfactory explanation of these contradictory facts could be

given. Cutler [1930], however, has reported that a number of organisms capable of producing nitrite from ammonia have been isolated from Rothamsted soil and percolating filters similar to the one described in this paper. Runov [1926, 1928] and Mischustin [1926, 1928], described nitrifying bacteria which multiply in media containing organic matter. The discovery of other organisms which transform nitrites into nitrates in the presence of organic matter therefore appears to await the solution of a suitable technique for their growth and isolation. The experiments described in this paper suggest the existence of such organisms.

SUMMARY.

1. The experiments described have been carried out with a percolating filter. This consisted of six independent sections, each 6 inches diameter and 9 inches deep, and filled with 1/8 inch to 1/4 inch clinker.

2. When a mixture of 0.033 % sucrose, 0.033 % acetic acid and 0.033 % lactic acid is fed to the filter described in (1) at a suitable rate of flow, it is readily oxidised by a mixed microbial population. A biological film is built up and the organic matter is oxidised even if the C : N ratio of the nutrient solution is 80 to 1.

3. If a 0.1 % solution of sucrose is allowed to trickle through a mature portion of the sectional percolating filter, some formation of acid and invert sugar takes place. For acid to be produced the p_H of the nutrient solution must not be less than 6.7 to 6.8. The maximum p_H drop recorded has been from an initial p_H of 7.0 to one of 6.6. The nature of the acid product is as yet unknown. It is suggested that the acid arises either as a normal intermediate product of sucrose oxidation which is destroyed almost instantaneously, or that its presence is due to the growth of a bulky film within which an acid, anaerobic fermentation occurs.

4. When the sectional filter was working well within its capacity, *i.e.* more than able to oxidise the organic matter with which it was supplied, it removed sucrose from a 0.1 % solution in approximately the following manner: Section No. 1, 40 %; No. 2, 25 %; No. 3, 12 %; No. 4, 10 %; No. 5, 7 %; No. 6, 6 %; total 100 %.

5. Each section of the percolating filter may be replaced by any other without the filter losing in efficiency. In fact the total working capacity of the filter could probably be increased by making each section the first in turn for a few weeks.

6. Oxidation of ammonia proceeds best in the lower sections where the concentration of sugar is least. It is not inhibited by concentrations of sugar up to 0.05 to 0.06 %. Therefore nitrification and carbohydrate oxidation take place simultaneously in several of the sections. Ample evidence exists in support of the view that bacteria which nitrify ammonia may be active in presence of organic matter.

7. Both nitrites and nitrates are produced by micro-organisms in the presence of 3 to 4 parts per 100,000 of nitrogen as ammonia.

8. Each section, irrespective of its previous function, rapidly shows nitrifying activity when placed in a suitable part of the filter.

The unpublished results referred to in the Introduction were obtained by Messrs E. H. Richards, R. B. Dawson, S. W. Johnson, J. T. Martin and the author. Mr E. H. Richards, Head of the Fermentation Department, has been personally responsible for the direction of the work described in this paper and his keen interest and stimulating criticisms have been highly valued.

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REFERENCES.

- Adeney (1908). *Royal Commission on Sewage Disposal* (Cd. 4284), p. 17. (H.M. Stationery Office, London.)
- Beijerinck (1914). *Folia Microb.* **3**, 91.
- Clifford (1907). *J. Soc. Chem. Ind.* **26**, 739.
- Cutler (1930). *Nature*, **125**, 168.
- Department of Scientific and Industrial Research (1928, 1929, 1930). *Annual Reps. of the Water Pollution Research Board*. (H.M. Stationery Office, London.)
- Foreman (1928). *Biochem. J.* **22**, 208.
- Fürth and Lieben (1922). *Biochem. Z.* **128**, 144.
- Hanes (1929). *Biochem. J.* **23**, 99.
- Ministry of Health (1929). *Methods of analysis as applied to sewage and sewage effluents* (H.M. Stationery Office, London.)
- Mischustin (1926). *Ber. bact. agron. Station (Moscow)*, **24**, 200.
- (1928). *Ber. bact. agron. Station (Moscow)*, **25**, 28.
- Runov (1926). *Ber. bact. agron. Station (Moscow)*, **24**, 189.
- (1928). *Ber. bact. agron. Station (Moscow)*, **25**, 40.
- Winogradsky (1891). *Ann. Inst. Pasteur*, **5**, 92, 577.

XXI. NOTES ON THE HAGEDORN-JENSEN METHOD FOR THE DETERMINATION OF BLOOD-SUGAR.

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THE Hagedorn-Jensen method for the determination of blood-sugar is frequently used not only in clinical blood analysis but also in research work. Most authors employing the method consider it quite satisfactory and accurate. Their criterion is the fact that the method yields very good duplicates. A few critical studies, however, cast some doubt upon its reliability. Thus, Duggan and Scott [1926] point out that since 0.01 cc. of 0.005 *N* thiosulphate is equivalent to 1.8 mg. of glucose per 100 cc. of blood, very slight differences in titration may cause considerable errors. This and the fact that the method employs 0.1 cc. of blood represent *a priori* limitations of accuracy. Folin and Malmros [1929] call attention to a more serious inherent source of error. They state that the cotton plug used for filtration, if too small and loose, allows some precipitate to pass through, rendering the results too high. If, on the other hand, the plug is larger and compact, it retards filtration and retains some filtrate, causing loss of sugar.

As a first step in our examination of the Hagedorn-Jensen method we performed numerous determinations, strictly following the original directions, on blood samples containing known amounts of glucose. These were prepared according to Somogyi [1930] by the addition of glucose to blood samples which were rendered sugar-free either by treatment with washed yeast or by glycolysis. Although duplicate determinations as a rule showed very good agreement, the quantities of the glucose recovered differed greatly from the amounts actually added. This points emphatically to the fallacy of the assumption that good checks between duplicate determinations may be considered as a criterion for the adequacy of a method. The criterion we have adopted in this work is the accuracy with which known amounts of glucose, added to sugar-free blood, can be recovered. The Hagedorn-Jensen method, as we shall presently show, proved to be entirely unreliable in such a test.

Next, we examined to what extent the source of error, pointed out by Folin and Malmros, may account for the faulty results. To determine the amount of sugar retained by the cotton plugs, after filtration and two washings,

¹ Working with a grant from the Selma Michael fund.

two or three plugs from parallel determinations were united and extracted with 12 cc. of water; the extract was filtered through a washed filter-paper, and the reduction of the filtrate was determined. Having found that cotton gives up reducing substances to water in measurable amounts, we employed in these experiments cotton which had been carefully washed and dried at room temperature. The results, given in Table I, show that 4 to 22 mg. per 100 cc. of the sugar is lost in the cotton filters, amounts of the same order of magnitude as Folin and Malmros found by a different technique.

Table I. *Showing that the cotton filters in the Hagedorn-Jensen method retain appreciable portions of the blood-sugar.*

No.	Blood-sugar in Hagedorn-Jensen filtrates mg. per 100 cc.	Blood-sugar retained in cotton filter mg. per 100 cc.
1	24	4
2	54	5
3	123	7
4	137	7
5	151	22
6	182	11
7	213	14
8	217	8
9	224	22
10	318	10

The magnitude of the loss depends largely upon the concentration of the blood sugar, assuming uniform bulk and density of the filter plugs. In the hands of one and the same worker the method may thus yield good duplicate determinations, while the results do not represent the actual total of the reducing substances.

Before carrying our investigations further we had to eliminate this source of error. To this end we used the Hagedorn-Jensen method in the following form. In a test-tube marked at 12 cc., 0.12 cc. of blood was precipitated according to the directions of Hagedorn and Jensen. After heat-coagulation the mixture was cooled and diluted to 12 cc. The test-tube was stoppered and well shaken, and the contents were filtered through dry filter-paper. Filter-paper thoroughly washed with slightly acidified water was used in this work. For the rest, the original procedure was followed using 2 cc. of the Hagedorn-Jensen ferricyanide reagent and 10 cc. of filtrate which corresponds to 0.1 cc. of blood. In order to prevent changes in the alkalinity of the reagent, the filtrates were always neutralised with 0.30-0.35 cc. of 0.1 *N* sodium hydroxide before the addition of the ferricyanide.

For the calculation of results we have prepared our own table with standard solutions of glucose. The original table cannot be used in accurate work, since Hagedorn and Jensen erroneously postulated that the self-reduction of ferricyanide reagents is always the same, and used a constant value for this as a correction. The fact is however that it is almost impossible to prepare two batches of Hagedorn-Jensen reagent that show the same self-reduction, and

the latter will change even in the same batch upon standing for some time. Instead we follow the procedure employed in other iodimetric methods and subtract the titration figures from the titration value of a "blank" sample of the reagent. The blank, in which water takes the place of the sugar solution, is treated exactly as the unknown. In this way self-reduction is ruled out and the difference value is equivalent to the reduction of the sugar solution. It is probably due to the arbitrary method of correction that Hüst and Hatlehol [1920] also found the sugar table of Hagedorn and Jensen unsatisfactory and felt the necessity of preparing one of their own. In Table II are presented the basic figures of our table.

Table II. *Glucose equivalents of titration values in the Hagedorn-Jensen method.*

Actual amount of glucose mg.	0.005 N thiosulphate (blank titration) · cc.	Blood-sugar if filtrate from 0.1 cc. of blood is used mg. per 100 cc.
0.01	0.07	10
0.02	0.12	20
0.05	0.26	50
0.10	0.53	100
0.15	0.80	150
0.20	1.07	200
0.25	1.33	250
0.30	1.60	300
0.35	1.87	350

Owing to the excellent proportionality between sugar concentration and reduction, a detailed table can easily be prepared by interpolation. Well reproducible results are obtained only up to a concentration of 350 mg. per 100 cc. blood-sugar; above this limit the values become unreliable. This difficulty we have obviated by increasing the concentration of the ferricyanide for the determination of higher concentrations of sugar. As to the lower limit, our experience agrees with that of Duggan and Scott [1926] who were unable to obtain good results below the 25 mg. per 100 cc. concentration of blood sugar (0.025 mg. of glucose).

In Table III are given examples of comparative sugar determinations by

Table III. *Showing that the original Hagedorn-Jensen method yields too low results for true blood-sugar.*

No.	True sugar* found Hagedorn-Jensen method		Amount of added sugar mg. per 100 cc.
	Original mg. per 100 cc.	Modified mg. per 100 cc.	
1	89	104	100
2	126	155	150
3	154	160	160
4	172	189	190
5	173	201	200
6	178	199	200
7	298	301	300
8	487	498	500

* True sugar = total reduction (apparent sugar)—reduction of sugar-free blood.

the original Hagedorn-Jensen method and by the technique eliminating the cotton filter. The figures represent true sugar values, obtained as the difference between total reduction and non-fermentable reducing substances. The latter were determined in the blood, after it had been rendered sugar-free by means of glycolysis or by treatment with washed yeast. To another portion of this sugar-free blood a known amount of glucose was added, and the total reduction was determined. The results obtained by the original Hagedorn-Jensen methods were computed from the original table of these authors, while those determined with our modification were read from our own table (Table II). As can be seen, the original Hagedorn-Jensen technique yields results that are too low, the discrepancies being rather irregular. From the figures of Tables I and III, it is apparent that the discrepancies are of the same order of magnitude as the losses of sugar in the cotton plugs, which shows that the technique of filtration is the essential source of inaccuracy, which renders the Hagedorn-Jensen method altogether inadequate for accurate analytical work. With the elimination of the cotton filter, and with a logical method of correcting for the self-reduction of the ferricyanide reagent, it is possible to obtain very satisfactory results.

This observation is entirely contradictory to all reports from other workers. Comparative blood-sugar determinations on record, carried out by the Hagedorn-Jensen method and several copper-reduction methods (Folin, Shaffer-Hartman, *et al.*) do not show appreciable or consistent differences in one direction or the other [Duggan and Scott, 1926; Van Slyke, Hiller and Linder, 1925; Höst and Hatlehol, 1920]. The apparent conflict however is readily explained by the fact that these authors determined apparent sugar values (total reduction), while we are dealing with true (fermentable) sugar. Somogyi has repeatedly pointed out [1930] that different reagents yield different values for the non-sugar reducing substances, and furthermore that different protein precipitants leave different quantities of these substances in the blood filtrates [Somogyi and Kramer, 1928]. In particular, ferricyanide gives much higher values for the non-fermentable reducing substances than do the copper reagents. We can corroborate the findings of Somogyi that the amount of the non-sugar reducing substances in the Hagedorn-Jensen method is 20-30 mg. per 100 cc., in the Folin-Wu method 12-15 mg. per 100 cc., in terms of glucose. The higher value in the Hagedorn-Jensen method obviously balances the loss of sugar in the cotton filter, thus concealing this serious source of error, and as a consequence the two methods frequently yield rather similar results. Comparison of total reduction instead of true sugar values, of course, could not disclose that fermentable sugar is actually lost in the Hagedorn-Jensen method.

In recent very careful experiments Herbert and Bourne [1930] found that the Hagedorn-Jensen method gives sugar values "slightly higher" than the true sugar obtained by Somogyi's method. The discrepancies vary between 1 and 10 mg. per 100 cc. According to our findings, such agreements are

possible only through coincidences in some cases in which the amounts of the reducing non-sugars, included in the Hagedorn-Jensen reduction values, happen to be the approximate equivalents of the amounts of sugar lost in the cotton filters. Since both the positive and the negative error are variable, the approach to true sugar values is bound to be quite inconsistent, and the discrepancies are frequently too great to be ascribed simply to "experimental errors."

SUMMARY.

An examination of the Hagedorn-Jensen method for the determination of blood-sugar reveals sources of error which render it inadequate for accurate work.

The method does not yield true sugar values, the equivalent of 20 to 30 mg. per 100 cc. of sugar being derived from reducing substances other than sugar. At the same time the results are considerably lower than would be expected, owing -- as Folin and Malmros have shown before -- to a retention of 4-22 mg. per 100 cc. of fermentable sugar in the cotton plugs employed for filtration.

The table of Hagedorn and Jensen, given for the computation of sugar, leads to an additional error. In this table a definite correction for the self-reduction of the alkaline ferricyanide reagent is applied, implying the assumption that self-reduction is a constant value for every reagent. A correction value of general validity however is impossible, as the self-reduction changes with time even in the same reagent.

REFERENCES.

- Duggan and Scott (1926). *J. Biol. Chem.* **67**, 287.
Folin and Malmros (1929). *J. Biol. Chem.* **83**, 121.
Herbert and Bourne (1930). *Biochem. J.* **24**, 299.
Host and Hatlehol (1920). *J. Biol. Chem.* **42**, 347.
Somogyi (1930). *J. Biol. Chem.* **86**, 655.
— and Kramer (1928). *J. Biol. Chem.* **80**, 733.
Van Slyke, Hiller and Linder (1925). *J. Biol. Chem.* **64**, 625.

XXII. THE VAPOUR PRESSURE DEPRESSIONS OF AQUEOUS SOLUTIONS OF PHOSPHATE BUFFER MIXTURES AT 20.3°.

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THE difficulties attendant upon the determination of the colligative properties of solutions, at temperatures other than the freezing and boiling points, have prevented the accumulation of data concerning these important physico-chemical functions. The direct measurement of osmotic pressure has been restricted almost entirely to a few organic substances, while accurate determinations of vapour pressure lowerings have been limited owing to the complexity of the methods and apparatus involved in the static and dynamic methods used heretofore. The recent introduction by Hill [1930, 1] of a thermal method for measuring vapour pressure, owing to its simplicity and ease of manipulation compared with the static or dynamic method, renders the determination of such colligative properties relatively easy. The method is particularly useful and accurate for the solution of many chemical problems, furnishing as it does a knowledge of a function whose thermodynamic relationship to other physico-chemical properties is well established.

The common use of Sørensen's [1912] phosphate mixtures as buffer solutions in biochemical work renders a knowledge of their colligative properties of great practical importance. Previous determinations of such properties have been limited to the freezing and boiling points [International Critical Tables, 1928] of solutions of the pure salts, no accurate data being available, even at these temperatures, for mixtures. Such data, therefore, at the suggestion of Mr G. S. Adair, have been obtained and are given below.

The vapour pressure lowerings recorded here were obtained by Hill's thermal method as described in previous papers from this laboratory [Hill, 1930, 1, 2; Margaria, 1930]. The same apparatus and general procedure were used as described elsewhere [Grollman, 1931]. The salts employed were standard "analytical reagents." The primary potassium phosphate (KH_2PO_4) was dried at 110° for a day before use. The secondary sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) was obtained by exposing $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ to the atmo-

¹ John Simon Guggenheim Memorial Fellow.

sphere, as suggested by Clark [1928]. The various buffer mixtures were prepared according to the usual procedures [Clark, 1928].

The results are given in Table I. Columns 1 and 2 of this table give the

Table I. *The vapour pressures of Sørensen's phosphate buffer mixtures at 20.3°.*

Composition of solution		p_H	NaCl concentration isomolar with solution. Moles per 1000 g. of water	Molar relative vapour pressure lowering $3.18 \pm 0.008 \times 10^{-2}$
Primary phosphate cc. of $M/15$ KH_2PO_4	Secondary phosphate cc. of $M/15$ Na_2HPO_4			
10.0	0.0	—	0.0637	$3.18 \pm 0.008 \times 10^{-2}$
9.5	0.5	5.589	0.0637	3.18 ± 0.013
9.0	1.0	5.906	0.0639	3.19 ± 0.004
8.0	2.0	6.239	0.0643	3.21 ± 0.016
6.0	4.0	6.643	0.0657	3.28 ± 0.008
4.0	6.0	6.979	0.0680	3.39 ± 0.015
2.0	8.0	7.381	0.0698	3.48 ± 0.010
1.0	9.0	7.731	0.0714	3.56 ± 0.013
0.5	9.5	8.043	0.0737	3.67 ± 0.014
0.0	10.0	—	0.0743	3.70 ± 0.016

composition of the various mixtures investigated. Column 3 shows the p_H of such mixtures, according to Clark [1928]. In column 4 is given the sodium chloride concentration isomolar¹ with the solution in question as found by experiment. Column 5 shows the relative molar vapour pressure lowering as defined by the expression $\frac{p_0 - p}{Mp_0}$, in which p_0 is the vapour pressure of pure water at the temperature of the experiment, p the vapour pressure of the solution, and M the number of moles of solute present per litre of solution. The value of M is constant for the solutions studied and equal to 1/15. It should be noted that the above described mode of expression for the molar relative vapour pressure lowering differs slightly from that usually employed, in which M is expressed in terms of the number of formula weights of solute present per 1000 g. of water. The former mode of expression has been used because buffer solutions are generally expressed in terms of volume concentrations.

The vapour pressure lowerings for pure Na_2HPO_4 and KH_2PO_4 solutions, as given in the table, were obtained by comparison with a 0.064 molar NaCl solution whose molar relative vapour pressure lowering was taken as 3.33×10^{-2} , as previously found [Grollman, 1931]. The data for the mixtures were then obtained by comparison with the Na_2HPO_4 or KH_2PO_4 solutions. The values quoted are the averages of at least four sets of determinations (8 readings) and the probable errors of the mean molar relative vapour pressure lowerings are included in the last column.

It might have been expected that the vapour pressure lowering would be a linear function of the composition of the mixture. This would be the case were no interaction to occur between the constituent salts, *i.e.* were the total

¹ This term is used to describe a solution of the same vapour pressure as a given solution under the same condition of temperature, etc.

ionic activity of the solute to remain constant. This expectation is not fulfilled, as is seen in Fig. 1.

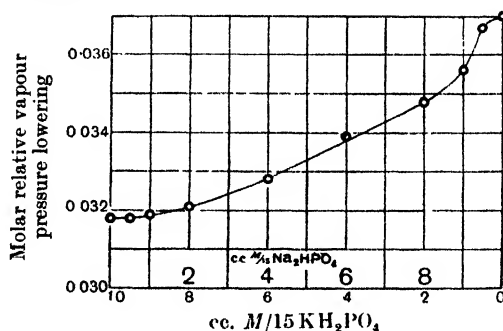


Fig. 1.

Ordinate. The relative molar vapour pressure lowering $(p_0 - p)/Mp_0$, where p_0 is the vapour pressure of pure water, p the vapour pressure of the solution, and M is the number of g. formula weights of solute per litre of solution.

Abscissa. The composition of the solutes expressed as cc. of $M/15 \text{ Na}_2\text{HPO}_4$ in 10 cc. of the mixture.

It is often assumed in biochemical and physiological work that the colligative properties of mixtures, such as are continually encountered in biological materials, are simple additive properties of the constituents. The inadequacy of this view, even where no apparent interaction occurs between the various solutes (in which case, of course, no such additive relationship would be expected to hold), has been shown elsewhere [Grollman, 1931]. The curve of Fig. 1 emphasises again the extent of the deviation of the colligative properties of a mixture from those anticipated on the assumption of a simple additive relationship between the properties of the mixture and those of its constituents.

The vapour pressure values quoted in the present paper, being functions of the total ionic activities of the solutions, may be utilised in calculations involving expressions for such activities.

SUMMARY.

Data are presented for the vapour pressure depressions of $M/15$ aqueous solutions of KH_2PO_4 , of Na_2HPO_4 , and of mixtures of these salts.

I am indebted to Professor A. V. Hill, at whose suggestion these measurements were made, for his constant interest and help. The work was carried out during the tenure of a John Simon Guggenheim Memorial Fellowship.

REFERENCES.

- Clark (1928). The determination of hydrogen ions, 3rd ed.
- Grollman (1931). *J. Gen. Physiol.* (In the Press.)
- Hill (1930, 1). *Proc. Roy. Soc. Lond. A* **127**, 9.
- (1930, 2). *Proc. Roy. Soc. Lond. B* **106**, 477.
- International critical tables of numerical data (1928). **3**.
- Margaria (1930). *J. Physiol.* **70**, 417.
- Sørensen (1912). *Ergebn. Physiol.* **12**, 393.

XXIII. OXIDATION-REDUCTION POTENTIALS OF PNEUMOCOCCUS CULTURES.

II. EFFECT OF CATALASE.

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(Received January 1st, 1931.)

IN previous communications the electrode potential behaviour of haemolytic streptococci [Hewitt, 1930, 1], *C. diphtheriae* [1930, 2], staphylococci [1930, 3] and pneumococci [1930, 4] has been described by the author and it has been shown that the oxidation-reduction potentials established in bacterial cultures depend upon the cultural conditions and upon the organism studied. The potentials developed in streptococcal cultures are roughly similar to those in pneumococcal cultures, but are quite different from those in staphylococcal and *C. diphtheriae* cultures.

Haemolytic streptococci and pneumococci form peroxide in aerated cultures, whilst staphylococci and *C. diphtheriae* do not, and it is becoming increasingly evident that peroxide formation is an important factor in bacterial behaviour. McLeod and Gordon [1923] classify bacteria on the basis of peroxide formation and account for the phenomenon of anaerobiosis on the grounds of the toxicity of peroxide. In his recent work on the effect of oxidation-reduction conditions on bacterial variation Todd [1930] finds that peroxide may have a selective inhibitory effect on a particular variant and thus peroxide formation may lead to selection of another variant less sensitive to peroxide.

In view of the importance of peroxide it is of interest to eliminate its effect and to determine what other differences exist between different bacteria. Addition of catalase to a peroxide-forming organism such as the pneumococcus should inhibit peroxide formation and thus reveal other characteristics in its oxidation-reduction behaviour.

In this paper are described the effects of catalase on the electrode potentials developed in pneumococcus cultures.

METHODS.

The general methods and apparatus for the measurement of electrode potentials were the same as those previously described [1930, 1]. An inoculum of 0.1 cc. of a 24-hour broth culture of pneumococcus type II was made into 8 cc. of medium in each case and the potential was observed from the time

of inoculation, at frequent intervals for the first 12 hours and then less frequently. The incubator temperature was 37°.

Catalase preparations.

Liver catalase. The liver catalase preparation was made by the general method of Batelli and Stern [1904]. Fresh liver was minced finely, extracted with water, the cell debris was centrifuged down and the supernatant fluid was treated with 2 volumes of 98 % alcohol. The flocculent precipitate was filtered off, dissolved in water and reprecipitated with alcohol. The precipitate was filtered off on a Büchner funnel, drained thoroughly and dried *in vacuo* over sulphuric acid. The yield was approximately 1 g. of dried powder from 1 lb. of fresh liver, and the preparation showed marked catalase activity.

Bacterial catalase. The bacterial catalase, kindly provided by Dr E. W. Todd, was prepared as follows. *Micrococcus lysodeikticus*, grown on agar slopes for 48 hours, was suspended in saline and rendered sterile by incubation with chloroform, the chloroform was removed and the killed organisms were then centrifuged down, the supernatant fluid being discarded. The freshly centrifuged cells had only a weak catalase activity but when allowed to stand overnight the catalase activity had increased enormously. Possibly the intact cells react sluggishly with hydrogen peroxide and catalase escapes slowly either by diffusion or by lysis of the cells.

Blood catalase. Sterile, washed red blood corpuscles suspended in saline were used as a third source of catalase.

RESULTS.

Liver catalase.

0.1 g. of the liver catalase preparation described above was dissolved in 10 cc. of water and filtered through a sterile Seitz filter. 1 cc. of this sterile 1 % solution was added to each 10 cc. of peptone infusion broth. Catalase broth was prepared by heating broth in a boiling water-bath for 30 minutes, cooling and adding the liver catalase, and inactivated catalase broth was prepared by adding the liver catalase and then heating the broth for 30 minutes. The catalase broth had marked catalase activity whilst the inactivated catalase broth, used in control experiments, had no catalase activity.

Aerobic cultures. In ordinary stationary aerobic cultures the potential fell to the same extent in the liver catalase broth and inactivated catalase broth. For the first 12 hours the catalase had no effect on the potential-time curve (Fig. 1). After the logarithmic phase of growth the potential of the culture in the inactivated catalase broth began to rise as is usual in pneumococcus cultures [Hewitt, 1930, 4] and the 48-hour culture had a potential 0.32 v. less negative than the 12-hour culture. In the catalase broth culture, on the other hand, the potential remained at the same low (*i.e.* reducing) level long after the logarithmic phase of growth and at the end of 50 hours had not

risen above the level of the 12-hour culture. The presence of catalase therefore prevents the disappearance of reducing conditions usually observed after the logarithmic phase of growth, although it has no effect on the initial establishment of reducing conditions. It seems evident that peroxide formation must be responsible for the disappearance of reducing conditions and the rise of potential since these are prevented by the presence of catalase. That peroxide formation is effective at such low (reducing) levels of potential is somewhat surprising and will be commented upon later.

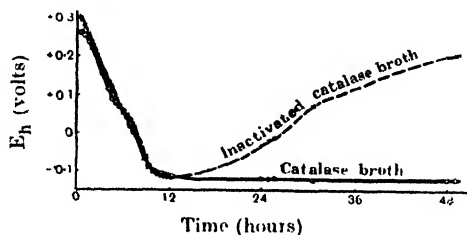


Fig. 1. Aerobic peptone infusion broth cultures of pneumococcus (liver catalase).

Aerated cultures. It was shown in a previous paper [1930, 4] that when cultures of pneumococci were subjected to vigorous aeration, the potential fell only to a slight degree and then rapidly rose to the high level of potential which corresponds with the formation of detectable amounts of peroxide. It was found that inactivated liver catalase had no effect on the electrode potentials of aerated pneumococcus cultures. In Fig. 2 the upper curve

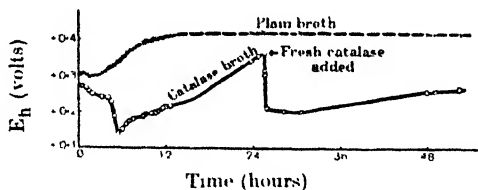


Fig. 2. Aerated pneumococcus cultures (liver catalase).

represents an aerated broth culture of pneumococci, and a similar culture containing inactivated catalase gave an almost indistinguishable curve. The potential fell only 0.007 v. then rose rapidly, and in 10 hours reached $E_h = +0.4$ v. at which level peroxide may be detected chemically in the culture. It was found, however, that in a similar culture containing active liver catalase the potential fell 0.13 v. and then commenced to rise. After 25.5 hours the potential had reached $E_h = +0.36$ v. At this stage a further quantity of liver catalase was added (equal in amount to that originally added). This resulted in an immediate rapid drop in potential amounting to 0.15 v. in 40 minutes. This effect was evidently due to decomposition of the peroxide present by the fresh catalase added, the original catalase having been destroyed by the continuous production of peroxide by the pneumococci.

A further interesting observation was made with regard to the effect of catalase. After 53 hours' incubation the aerated broth culture, the aerated inactivated catalase broth culture and the aerated catalase broth culture, were subcultured on blood agar. After 18 hours' incubation there was no growth from the two former inoculations but copious growth from the catalase broth culture. Peroxide formation had sterilised the broth cultures but the presence of catalase had protected the catalase broth culture. It is worthy of note that the aerated catalase broth culture was markedly turbid after 53 hours' growth showing that pneumococci flourish when the oxygen supply is abundant, provided that the harmful effects of peroxide are removed. This confirms the view previously expressed [1930, 1, 2, 3] that many bacteria grow most luxuriantly in the presence of a generous oxygen supply. This effect may be obscured with organisms which do not contain catalase owing to the toxic effect of the peroxide which may be formed when the oxygen supply is abundant.

Bacterial catalase.

0.05 cc. of a sterile killed preparation of *M. lysodeikticus*, centrifuged down to a thick suspension, was added to each 10 cc. of broth. For bacterial catalase broth the broth was heated for 30 minutes in a boiling water-bath and cooled before the addition of the preparation whilst for inactivated bacterial catalase broth, used as a control, the preparation was added to the broth before heating.

Aerobic cultures. In ordinary stationary aerobic cultures the effect of bacterial catalase was similar to that observed with liver catalase (Fig. 3).

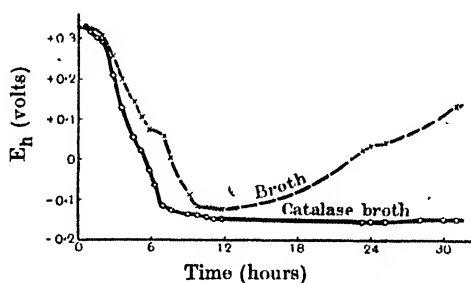


Fig. 3. Aerobic pneumococcus cultures (bacterial catalase).

After 30 hours' incubation the culture containing bacterial catalase had the same low potential reached after 12 hours' growth whereas in a similar culture not containing catalase the potential had risen 0.16 v. It may be worthy of note that whereas the boiled inactivated suspension of *M. lysodeikticus* had no effect on the establishment of reducing conditions, the unheated but killed bacteria appeared to facilitate reduction, since the potential fell rather more rapidly and to a slightly lower level in the bacterial catalase broth. This effect was not seen with liver catalase nor with blood catalase and may possibly be due to certain enzymes or accessory substances in the killed bacteria. The

point of inflection on the plain broth curve just below $E_h = +0.1$ v. has been observed on a number of occasions but its significance is uncertain.

Aerated cultures. Bacterial catalase had the same effect as liver catalase on the electrode potential behaviour of vigorously aerated cultures of pneumococci (Fig. 4). In the aerated broth and the aerated inactivated bacterial

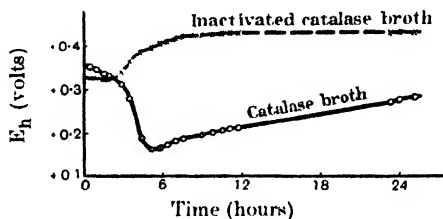


Fig. 4. Aerated cultures (bacterial catalase).

catalase broth cultures the potential fell only 0.004 to 0.007 v. and then rose fairly rapidly reaching the peroxide level ($E_h = +0.4$ v.) after 6 hours' incubation. In the bacterial catalase broth, however, the potential fell to the extent of 0.19 v. and then rose very slowly and no peroxide was formed even after 30 hours' incubation.

Blood catalase.

Blood catalase broth was prepared by adding 0.1 cc. of sterile washed red blood corpuscles to each 10 cc. of broth.

Stationary aerobic cultures. In stationary aerobic cultures containing blood catalase the potential fell in the usual way except that a flat portion of the curve occurred at a level of $E_h = +0.3$ v. (Fig. 5). The form of the curve suggested that some poisoning oxidation-reduction system had to be reduced before the potential could fall. It seems probable that some haemoglobin system may be responsible for this effect. In this connection it may be mentioned that only reversible oxidation-reduction systems can give a well-defined

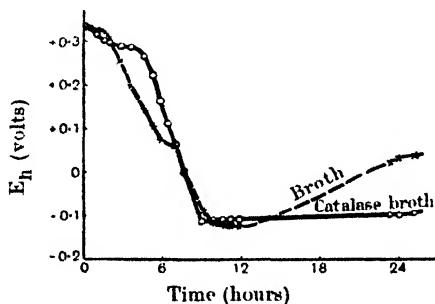


Fig. 5. Aerobic pneumococcus cultures (blood catalase).

potential at an unattackable electrode, but other systems may affect the potential-time curve of a culture and have a poisoning effect, since other reducing actions (which would be reflected in the fall in potential) cannot occur until the poisoning system is itself reduced.

The potential in a stationary aerobic blood catalase broth culture of pneumococci remained at a low level after 30 hours' incubation, whereas the potential in a plain broth culture had risen in the usual way. Blood catalase thus had the same effect in maintaining reducing conditions after the cessation of active proliferation as had liver and bacterial catalase.

Aerated cultures. The flat portion of the curve which was observed in stationary cultures was noticed also in vigorously aerated blood catalase broth cultures (Fig. 6). In the presence of blood catalase the potential of aerated

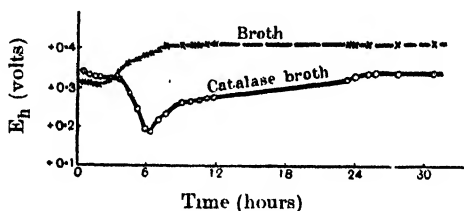


Fig. 6. Aerated pneumococcus cultures (blood catalase).

cultures fell to a much greater extent and rose more slowly than in plain broth cultures. Thus blood catalase had the same general effect on the potential as had liver and bacterial catalase preparations.

DISCUSSION.

The presence of catalase has very marked effects on the oxidation-reduction conditions of pneumococcus cultures. By using catalase from different sources—from liver, bacteria and blood—it is possible to eliminate the interfering effects inherent in the use of enzymes which cannot be isolated in the pure state but which are always contaminated with other substances.

In ordinary aerobic pneumococcus cultures the presence of catalase appears to have no effect on the establishment of reducing conditions during the phase of active proliferation, but after this phase the electrode potential in a plain broth culture commences to rise, whilst in the presence of active catalase the potential remains at a low level for a considerable time. It seems evident therefore that the usual rise in potential, after the logarithmic phase of growth of pneumococci, is due to peroxide formation, which evidently occurs long before peroxide can be detected chemically. The hydrogen peroxide formed in oxidation-reduction processes is, therefore, immediately decomposed by catalase, before it has time to affect the electrode potential, which remains at a low (*i.e.* highly reducing) level. The electrode potential behaviour of an aerobic pneumococcus culture containing catalase is therefore roughly similar to that of cultures of staphylococcus or *C. diphtheriae* which produce their own catalase.

In vigorously aerated pneumococcus cultures proliferation occurs very slightly, if at all, and the potential shows only a slight tendency to fall (0.004 to 0.007 v.) and then rapidly rises to the level at which peroxide may be

detected chemically. When catalase is present, however, the potential falls appreciably (0.13 to 0.19 v.) and rises only slowly. As long as active catalase is present the level corresponding to peroxide accumulation is not reached but the catalase present is gradually destroyed by the active oxidising system. When fresh catalase was added to an aerated culture from which the catalase had disappeared there was an immediate rapid drop in potential.

50-hour aerated broth cultures of pneumococci are sterile but in aerated cultures containing catalase the bacteria still proliferate actively when sub-cultured after 50 hours' incubation. Growth is negligible in aerated plain broth cultures of pneumococci but in aerated catalase broth cultures growth is very good, showing that pneumococci flourish when the oxygen supply is abundant provided that the toxic effect of peroxide is eliminated.

Although there is a considerable fall in potential (*circa* 0.15 v.) in aerated catalase broth cultures of pneumococci, this fall is much less than that observed with staphylococci and *C. diphtheriae* (0.35 to 0.45 v.). This suggests that there are differences in reducing power of different organisms quite apart from the catalase- and peroxide-forming functions. It is by no means certain, therefore, that the failure of obligate anaerobes to grow aerobically is due solely to potential peroxide production in the absence of catalase. Not only the toxic effect of peroxide but also inability to effect oxidation-reduction processes at high levels of electrode potential may account for the phenomena of anaerobiosis.

Bacterial peroxide and hydrogen peroxide have a number of properties in common.

(1) They give the same chemical reactions (*e.g.* the benzidine-peroxidase reaction);

(2) display similar electrode-potential behaviour [Hewitt, 1930, 1];

(3) are both decomposed in the presence of catalase.

These facts suggest that bacterial peroxide and hydrogen peroxide if not identical must be very closely related compounds, and my experiments, so far, have failed to reveal any differences between them.

Apart from their catalase effect *M. lysodeikticus* and red blood corpuscles have other effects on the oxidation-reduction conditions developed in pneumococcus cultures. Killed *M. lysodeikticus* appeared to accelerate reduction effects. This reducing effect was destroyed by heating and may be due to the presence of reducing enzymes or accessory substances. Red blood corpuscles, on the other hand, exerted a poisoning effect possibly due to some haemoglobin system which itself had to be reduced before further reducing effects could be developed in the culture.

SUMMARY.

1. Some of the characteristic differences in behaviour between pneumococci and catalase-forming bacteria disappear when catalase preparations are added to the pneumococcus culture.

2. When catalase has been added the electrode potential of aerobic pneumococcus cultures remains at a low level long after the logarithmic phase of growth.

3. In aerated cultures the potential falls to a much lower level and growth is much more luxuriant when catalase has been added.

4. Bacteria differ in oxidation-reduction behaviour in respects other than that of peroxide formation.

5. Bacterial peroxide and hydrogen peroxide possess many properties in common.

6. Indications were obtained of an acceleration of reduction effects by killed aerobic bacteria (*M. lysodeikticus*) and of a poisoning effect by erythrocytes.

The author is indebted to Dr R. G. White and Dr E. W. Todd for their continued help and encouragement.

REFERENCES.

- Batelli and Stern (1904). *Compt. Rend. Soc. Biol.* **57**, 374.
Hewitt (1930, 1). *Biochem. J.* **24**, 512.
— (1930, 2). *Biochem. J.* **24**, 669.
— (1930, 3). *Biochem. J.* **24**, 676.
— (1930, 4). *Biochem. J.* **24**, 1551.
McLeod and Gordon (1923). *J. Path. Bact.* **26**, 326.
Todd (1930). *Brit. J. Exp. Path.* **11**, 368, 469, 480.

XXIV. THE TEMPERATURE COEFFICIENTS AND ENERGY EXCHANGES OF THE CITRIC ACID DEHYDROGENASE OF CUCUMBER SEEDS.

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AMONG modern theories of chemical reaction the activation theory has proved to be one of the most fruitful not only in the hands of the physical chemists who were primarily responsible for its development, but also as a tool for the biochemist in his attempt to elucidate the mechanism of enzyme action. The theory postulates that an exceptionally high energy content is the most important of the criteria which determine the activated state preceding reaction, because the change of reaction velocity with changing temperature follows an expression of the same form as the law which expresses the variation with temperature of the number of molecules whose energy content exceeds an assigned value. The acceptance of this postulate has led to the view that in general the energy content of a molecule must be increased by an energy increment (the "critical increment" or "energy of activation") as a necessary prelude to chemical change of that molecule.

In terms of this theory the function of a catalyst is to aid the molecules of the reactant to attain the activated state—that is, to lower the critical increment. It is the examination of the way in which a heterogeneous catalyst lowers the critical increment of the molecules of a reactant which is germane to the problem of the mode of action of an enzyme. The action of the catalyst here can be described rather loosely as the straining of the substrate molecule, and this description has the merit of suggesting that the catalyst does work upon or yields energy to the molecule in activating it; but since it has become widely recognised that the forces in play at the boundary of any two phases are not purely physical but are chemical forces of valency, a more helpful picture of catalysis results from the legitimate postulate that the catalyst and reacting substance combine chemically, and that in the process the molecules of the reactant become activated.

This hypothesis may be applied to enzyme reactions in two ways. In the first method the measure of the critical increment may be ignored and attention may be concentrated on the changes wrought in the substrate molecule during activation, *i.e.* upon the straining of the molecule, which is most easily pictured as isomerisation. This mode of attack was employed by Quastel [1926]

and Quastel and Wooldridge [1927 and 1928] in studies of the enzyme systems of resting *B. coli*, although it is evident that Quastel did not regard the union of enzyme and substrate as a simple chemical reaction. The second method is to ignore the qualitative effect of activation on the substrate molecule and to examine the energy exchanges between the enzyme, the substrate and the surrounding system. This quantitative method has its own limitations—for instance it cannot lead us to a reason why citric acid dehydrogenase oxidises citric acid to acetonedicarboxylic acid while, in the absence of the dehydrogenase, reduction of the critical increment of citric acid by raising the temperature causes it to pass into aconitic acid—but it yields valuable data in other directions. The energy changes are measured indirectly by calculation from observations of the kinetics of the enzyme action, a fact of importance because at present the kinetics alone among the characteristic properties of an enzyme are amenable to exact quantitative measurement.

A rational description of the energy relations accompanying an enzymic reaction can be given on the assumption that the union of enzyme and substrate is a simple chemical reaction, following the hypothesis developed by Michaelis and Menten [1913] to account for the observed facts relating to the effect of substrate concentration on the rate of sucrose hydrolysis by saccharase, and subsequently applied with such marked success to the elucidation of many problems in the kinetics of this and of other enzymes. Their theoretical deduction of the relation between substrate concentration and initial reaction velocity for saccharase may be generalised in the following way. Let E represent the molecule of the enzyme, S the molecule of the substrate, and P the molecule of the product (or of one of the products if there are several). Then the enzyme and substrate are assumed to combine reversibly to form the compound ES which breaks down irreversibly into $E + P$, according to the equation $E + S \rightleftharpoons ES \rightarrow E + P$. At the commencement of reaction, the total molar concentrations of enzyme, substrate and the compound ES being represented by e , s and p respectively, the dissociation constant of the compound ES is given by

$$K_m = \frac{(s-p)(e-p)}{p} = \frac{s(e-p)}{p}$$

to a close approximation because s is supposed to be much greater than e ; and

$$p = \frac{es}{K_m + s}.$$

If the velocity constant of breakdown of ES to $E + P$ is written k , then the actual initial velocity of reaction

$$V = kp = \frac{k es}{K_m + s}.$$

This expression foretells the shape of curve obtained by plotting the initial velocity of reaction against substrate concentration to be a rectangular hyperbola, since V increases rapidly at first as s is increased from zero and then when s exceeds K_m further increase causes V to approach asymptotically the

limiting value $V_{\max} = ke$. Further, V will reach a figure equal to half the limiting value when $s = K_m$. Thus for an enzyme which follows the Michaelis equations, measurements of the initial rate of the reaction with varying substrate concentrations render it possible to calculate the value of the Michaelis constant K_m , which is one of the most characteristic constants of an enzyme. The assumption that the velocities of formation and dissociation of the enzyme-substrate compound are much larger than the velocity of its breakdown to the products of reaction is implicit in the above derivation; but there is evidence which suggests its validity [*cf.* Haldane, 1930, p. 41], and for the present it will be accepted as correct.

In considering the energetics of an enzyme action represented as above, there are three important and characteristic quantities which must be carefully distinguished. These are (1) the heat of formation of the compound ES , (2) the energy of activation, otherwise the critical increment, of the compound ES , which is necessary to enable it to react to yield the product P ; and (3) the heat of the reaction $S \rightarrow P$, which is the total heat of reaction of the complete process, and which is not of necessity related to the other heat quantities. The first quantity can be calculated when the equilibrium constant of the reaction $E + S \rightleftharpoons ES$ is known at two or more temperatures, with the aid of van't Hoff's reaction isochore

$$\frac{d(\log_e K)}{dT} = -\frac{Q}{RT^2},$$

where K is the equilibrium constant of the reaction, Q the heat of formation, T the absolute temperature, and R the gas constant. When an enzyme follows the Michaelis laws and the value of the Michaelis constant can be measured at different temperatures it is therefore possible to determine the heat of formation of the enzyme-substrate compound. The heat of activation of this compound can be calculated by the use of a similar expression due to Arrhenius, when the velocity constant k of its breakdown can be measured at two temperatures; and finally the heat of the catalysed reaction can be calculated by use of the isochore if the change of the total equilibrium with change of temperature is known. As the present considerations deal only with the initial stages of reaction this heat quantity is left out of account because it cannot be calculated from observations of the early stages of the reaction; attention has therefore been confined to the first two quantities and the relation between them.

The calculation of the first two of the separate heat quantities detailed above depends on the measurement of the temperature coefficients of the Michaelis constant and of the velocity constant k respectively, and since Haldane [1930, p. 38] has suggested that the characteristically low Michaelis constants of oxidising enzymes as compared with those of hydrolytic enzymes may indicate a fundamental difference between the two types, it is of immediate interest to discover whether the temperature coefficient of K_m is of the

same magnitude for enzymes of the two types¹. Before any generalisations can be made it will be necessary to obtain data about many enzymes, but as accurate data for the hydrolysing enzyme saccharase are already on record any figures for an oxidising enzyme will be of value for comparison. To obtain suitable data demands observations upon some typical oxidising enzyme, the desiderata which govern the choice being that the Michaelis constant is small and is susceptible of reasonably accurate measurement over some suitable temperature range.

Such an enzyme is the citric acid dehydrogenase described recently by Thunberg [1929]. Fig. 1 embodies data given by Thunberg in his paper, and

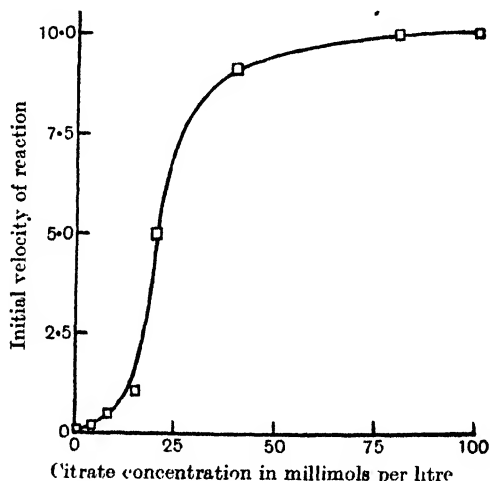


Fig. 1. The relation of initial velocity of reaction to substrate concentration for the citric acid dehydrogenase of cucumber seeds. Drawn from data given by Thunberg [1929].

it shows that the dehydrogenase has a low Michaelis constant (which can be read approximately from the graph as the substrate concentration at which the initial velocity reaches half its maximum value) and suggests that it should be possible to measure this constant with considerable accuracy. The curve deviates from the ideal rectangular hyperbola to a considerable extent at the lowest substrate concentrations because it is impossible with the technique available to measure at all accurately the initial rate of reaction with very small concentrations of substrate. The experimental method consists of the observation of the time taken for a standard small amount of the substrate to be oxidised, the concentration of the substrate at the commencement of the observation being known. It is thus implicitly assumed that the velocity throughout the observation remains constant at the initial value, and error is introduced because this can only be true with relatively high concentrations. Fortunately this difficulty vanishes if the lowest concentration at which

¹ Professor J. B. S. Haldane suggested this problem to the author, pointing out the importance which would be attached to the discovery of a high temperature coefficient of K_m for an oxidising enzyme.

accurate measurement of the reaction time is possible is less than the concentration at which the enzyme is half saturated by its substrate, because corrections can then be applied in order to calculate the true initial velocity from the apparent initial velocity of the reaction.

These considerations led the writer to attempt the measurement of the temperature coefficients of K_m and of the velocity of reaction for citric acid dehydrogenase in order to gain an insight into the energy exchanges occurring during its action.

EXPERIMENTAL METHODS.

All observations were made by the method of Thunberg [1928]. The dehydrogenase was allowed to act on its substrate in a Thunberg vacuum tube, and the hydrogen acceptor used was methylene blue. Each individual observation consisted in noting the time taken for the reduction of a standard amount of methylene blue under carefully controlled conditions of temperature, p_H , substrate concentration, enzyme concentration, etc.

The enzyme solutions employed in this work were prepared from seeds of the cucumber (*Cucumis sativa*) which were obtained from Messrs Weibull of Landskrona, Sweden, and bore the trade name "Västerås Grön Druv." For each series of observations a sufficient quantity of the seeds was shelled to give 5 g. of kernels, which were then ground in a mortar with 25 cc. of 0.87 % dipotassium hydrogen phosphate for 20 minutes. The milky liquid so obtained was centrifuged for 10 minutes at a speed of approximately 4000 r.p.m., at the end of which time it had separated into three layers. The bottom layer contained solid residue, the second layer which was still rather milky in appearance contained the active material, and the top layer consisted of the seed fats. The aqueous layer was separated from the others and was ready for immediate use.

In a single experiment a series of Thunberg vacuum tubes was prepared each containing 0.4 cc. of a solution of potassium citrate of known concentration. To each tube in turn were added 0.3 cc. of a 1 in 60,000 solution of methylene blue and 0.5 cc. of the enzyme solution; the tubes were then evacuated at the water-pump for 90 seconds, closed and immersed in a water-bath of which the temperature was kept constant within 0.05° . The concentrations of the potassium citrate solutions added at first were so graded that the complete reaction mixtures formed a series in which the citrate concentration varied from 4 mM to 400 mM, and other factors were constant. The time for the complete decoloration of the methylene blue in each tube was observed.

This method is similar in essentials to the one employed by Thunberg [1929] for the estimation of citric acid by means of this dehydrogenase, and the following comments may be made on it.

(1) As the total volume of reaction mixture in each tube is only 1.2 cc., it is essential that the tubes be thoroughly dried before use.

(2) The reaction is accelerated by fats and oils, so that the reaction mixture must not under any circumstances be allowed to come into contact with the grease lubricating the tap at the top of the tube. It is an advantage to put the whole of the enzyme preparation in a small Büchner flask and evacuate the vessel for a few minutes before making up the reaction mixtures, as the amount of frothing in the individual vacuum tubes during evacuation will be greatly diminished thereby. For the same reason the vacuum tube must be completely immersed in the thermostat. If the top of the tube is above the water level of the bath, distillation will occur within it and water from the reaction mixture will condense within the cooler tap and come into contact with the tap grease as it runs down the tube into the reaction mixture again. The reduction time of the methylene blue may be decreased by 50 % if this precaution is not taken.

(3) Evacuation by a good water-pump for 90 seconds reduces the oxygen concentration in the tube sufficiently.

(4) A fresh enzyme solution must be prepared for each series of observations, and kept at 0° if not used immediately.

(5) The measurements must be made at the optimum p_H of the system [cf. Nelson and Bloomfield, 1924].

(6) It was found that the results obtained always contained some irregular variations which could not be eliminated, therefore in the determination of the temperature coefficients the writer, after becoming conversant with the technique of the observations, performed a series of five experiments at 25° with different samples of active material, and from the readings calculated the mean value of K_m at that temperature. In the same way a mean value of K_m at 35° was measured and the temperature coefficient of the constant was determined from the two mean values.

EXPERIMENTAL RESULTS.

Using the enzyme in phosphate solution as described above, it was found that at the end of the period of observation the p_H of the reaction mixture as determined by means of the quinhydrone electrode was always 7.84 ± 0.03 . On account of the necessity of measuring the temperature coefficient at the optimum p_H of the system, a preliminary examination of the p_H -activity relationship of the dehydrogenase was made in which the initial velocity of reaction was determined at different hydrogen ion concentrations, all measurements being made with substrate concentrations high enough to ensure that the enzyme was fully saturated by its substrate. The activity was found to rise from a minimum in acid solutions (the enzyme was precipitated from solution below p_H 3.5) to a maximum at about p_H 7, and in more alkaline solutions the activity remained constant until a p_H above 10 was reached. This result is expressed in the curve shown in Fig. 2. All experiments carried out with the enzyme in dilute secondary phosphate solution were therefore done within the optimum activity range of the system.

The reduction time of the methylene blue in each of the tubes of all ten experiments is given in Table I. To calculate the value of K_m from the figures for each experiment it is first necessary to work out the initial velocity of reaction V at each concentration of citrate, relative to the initial velocity V_{\max} , which is attained when the citrate is sufficiently concentrated to saturate the

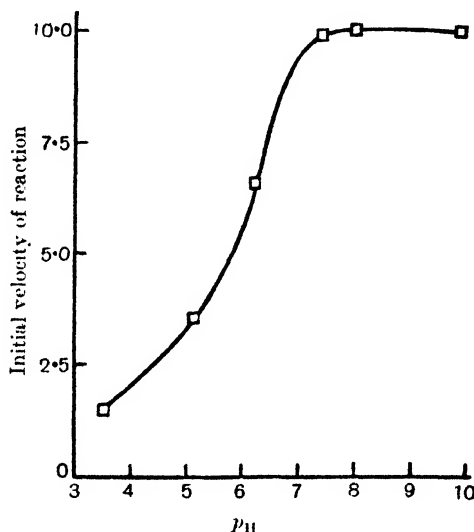


Fig. 2. The pH -activity curve for the citric acid dehydrogenase.

enzyme fully. The velocity of reduction of the methylene blue in each tube is proportional to the reciprocal of the reduction time, therefore the reciprocal of each of the reduction times recorded in each experiment has been tabulated and multiplied by 100 for convenience. The velocity of oxidation of the citrate in any tube is taken as proportional to the difference between the velocity of reduction of methylene blue in that tube and the velocity of reduction of methylene blue in the blank tube of the experiment (which contains no citrate). Finally, as in each experiment it was found that the initial velocity reached a maximum when the citrate concentration was 200 mM , the initial velocity at this concentration is taken as unity and the initial velocity at each of the other concentrations is expressed as a fraction of the initial velocity with 200 mM citrate, under the title $\frac{V}{V_{\max}}$. The successive figures obtained in this way from Exp. 1 are set out in Table II as an example of the procedure adopted to calculate values of $\frac{V}{V_{\max}}$ at each concentration in each experiment. In Table III the whole of the figures for $\frac{V}{V_{\max}}$ obtained in the complete series are collected together.

To find the heat of activation of the enzyme-substrate compound it was necessary to measure the reaction velocity with citrate concentrations large enough to saturate the enzyme completely; readings were taken both at 25°

Table I.

Reduction of methylene blue by citric acid in presence of citric acid dehydrogenase at p_H 7.84. Each tube contained 0.5 cc. of enzyme solution in 0.87 % secondary potassium phosphate, 0.4 cc. of potassium citrate solution of varying concentrations, and 0.3 cc. of a 1 in 60,000 solution of methylene blue.

Exp. No.	Temp.	Reduction time in minutes									
		120	115	92	92	100	63	32	27	23	27
1	25°	120	115	92	92	100	63	32	27	23	27
2	"	71	70	54	64	64	54	30	27	21	23
3	"	56	70	44	64	47	41	*	21	21	24
4	"	76	85	76	81	83	59	43	38	37	31
5	"	99	93	98	92	89	69	75	37	33	30
6	35°	61	60	60	53	58	41	24	26	26	24
7	"	52	55	50	43	41	36	22	22	23	22
8	"	64	64	44	*	54	35	22	19	19	19
9	"	76	72	64	66	54	49	26	24	22	22
10	"	71	65	55	57	59	46	31	29	27	26
Citrate <i>mM</i>		0	4	8	16	20	40	80	100	200	400

* Indicates no figure obtained.

Table II.

Calculation of $\frac{V}{V_{max}}$ at each concentration for Exp. 1.

Citrate <i>mM</i>	0	4	8	16	20	40	80	100	200
Reduction time minutes	120	115	92	92	100	63	32	27	23
$\frac{100}{\text{Reduction time}}$	0.83	0.87	1.09	1.09	1.0	1.58	3.11	3.7	4.35
$V = \frac{100}{\text{Reduction time}}$	-0.83	0	0.04	0.26	0.26	0.17	0.75	2.28	2.87
$\frac{V}{V_{max}}$	0	0.01	0.07	0.07	0.05	0.21	0.64	0.82	1.0

Table III.

Values of $\frac{V}{V_{max}}$ for all tubes of Exps. 1 to 10, calculated from the reduction times given in Table I.

Table 1.

Exp. No.	V								
	V_{max}								
1	0	0.01	0.07	0.07	0.05	0.21	0.64	0.82	1
2	0	0	0.12	0.04	0.04	0.12	0.55	0.65	1
3	0	0.12	0.16	0.08	0.11	0.22	—	1	1
4	0	0.1	0	0.05	0.06	0.23	0.74	0.98	1
5	0	0.03	0	0.04	0.06	0.22	0.57	0.81	1
6	0	0.01	0.01	0.11	0.04	0.37	1.15	1	1
7	0	0.04	0.03	0.16	0.2	0.35	1.08	1.08	1
8	0	0	0.2	—	0.07	0.35	0.81	1	1
9	0	0.03	0.09	0.07	0.18	0.25	0.86	0.97	1
10	0	0.06	0.18	0.15	0.13	0.33	0.80	0.89	1
Citrate mM	0	4	8	16	20	40	80	100	200

and at 35° with the same enzyme preparation. These measurements were made on tubes which contained 0.5 cc. of enzyme solution, 0.3 cc. of 1 in 60,000 methylene blue and 0.4 cc. of a solution of potassium citrate, which made the citrate concentration of the reaction mixture 400 *mM*. The ratio k_{35}/k_{25} was found to be 1.61. Repetitions of the observation with other enzyme preparations gave values of 1.70 and 1.64; and one observation over

the wider temperature range from 15° to 35° gave the figure 2.71 for the ratio of the maximum velocities of reaction at these two temperatures.

In addition to the measurement of the temperature coefficients, some observations on other qualitative and quantitative properties which have not yet been fully investigated may be reported briefly. The fatty layer which separates on centrifuging the cucumber seeds ground up in phosphate possesses the power of accelerating the action of the enzyme: a fact which has also been noted by Thunberg (private communication to the author). Citraconic acid accelerates the action slightly, but on the other hand aconitic acid inhibits competitively, just as was found for the citric acid dehydrogenase of liver by Bernheim [1928]. Finally it was noticed that under certain conditions the enzyme preparation is much more slowly destroyed on standing than is suggested by Thunberg [1929] and other workers of his school, *e.g.* Scherstén [1929]. This was investigated in order to ensure that the destruction of the enzyme at 35° was not so great as to cause serious error in the measurement of the temperature coefficients. In a typical experiment it was found that under the standard conditions specified above, 0.5 cc. of a freshly prepared enzyme solution caused the reduction of 0.4 cc. of 1 in 60,000 methylene blue in presence of 400 *mM* citrate in 49 minutes, while after the enzyme had been kept at 35° for one hour it brought about an identical reduction in 54 minutes. The destruction of the enzyme was therefore slight. As it is to be expected that the destruction in presence of the substrate occurs more slowly, the error so introduced into the measurement of the temperature coefficients is negligible.

Calculation of the energy exchanges.

(1) *The heat of formation of the enzyme-substrate compound.* The first step is to calculate the value of K_m at 25° and at 35°. From the data of Table III it can be seen that the figures for $\frac{V}{V_{\max}}$ are extremely scattered for concentrations less than 40 *mM* and cannot be used in the determination of the temperature coefficient. This indeed is to be expected because the concentration of methylene blue in the reaction mixtures is approximately 10 *mM* (reckoning the molecular weight as 400), and serious errors are introduced when the concentration of the methylene blue approaches that of the citrate. The figures for $\frac{V}{V_{\max}}$ are however definitely greater at 35° than at 25° for 40 *mM* and 80 *mM* citrate solutions; and the figures for 100 *mM* solutions show plainly that the enzyme is saturated by its substrate at lower concentrations of citrate at 35° than at 25°. The form of the calculation by which K_m is derived from the figures for $\frac{V}{V_{\max}}$ magnifies greatly the experimental errors if $\frac{V}{V_{\max}}$ is greater than 0.5, therefore the calculations which follow are based on the $\frac{V}{V_{\max}}$ figures for the 40 *mM* citrate solutions in each experiment.

Since at a substrate concentration s ,

$$V = \frac{kes}{K_m + s},$$

and since the limiting value $V_{\max.} = kc$, it follows that

$$\frac{V}{V_{\max.}} = \frac{s}{K_m + s},$$

and therefore

$$K_m = s \left(\frac{V_{\max.}}{V} - 1 \right).$$

From each experiment of the series K_m has been calculated by means of this formula, and the figures are given in Table IV. -

Table IV.

The Michaelis constants calculated from the data of Table III.

Exp. No.	1	2	3	4	5	6	7	8	9	10
K_m	150	293	142	134	142	68	74	74	120	81

Although these results are scattered, there is no overlapping of the figures for the first five experiments at 25° with those for the last five at 35°, and it is clear that there is a considerable shift of K_m due to a temperature change of 10°. The mean value at 25° is 172 and at 35° it is 83; a decrease which corresponds to a notable heat of reaction.

Before accepting these as the best available figures the errors they involve must be discussed. First there are the random errors of measurement which are partially eliminated by taking the mean values at each temperature and are probably quite small. Secondly, errors are introduced by a real difference between the Michaelis constants of different enzyme preparations. The figures for Exps. 2 and 9 vary from the means for their respective groups so much as to decide this point. Thirdly, in each figure is incorporated an error due to the relatively large concentration of methylene blue which it is necessary to use in the reaction mixture. The first and second errors are not serious because they affect the figures for K_m at both temperatures to the same extent, and will not therefore alter the ratio $K_m^{35^\circ}/K_m^{25^\circ}$ significantly. On the other hand, the third error varies in magnitude as the Michaelis constant changes, and as the change of Michaelis constant with temperature here recorded is much greater than has heretofore been discovered, it is necessary to show that the change cannot be due to this error distorting the results. The treatment which follows here shows that the error introduced is quite small, and although the true values of K_m are all rather smaller than those given in Table IV the ratio of the mean value at 35° to the mean value at 25° is almost unaffected.

The error is due to the fact that the concentration of the methylene blue is so large that it causes the citrate concentration to fall by an appreciable amount during the reaction. If t_0 be the reduction time of the blank tube and t_{40} the reduction time of the tube containing 40 mM citrate in any experiment, then in the tube containing the citrate the number of millimols of citrate reduced by the "blank process" is about $10 \times t_{40}/t_0$, since the initial concentration of methylene blue is approximately 10 mM. The number of millimols reduced by the citrate is therefore $10(1 - t_{40}/t_0)$, and the concentration of the citrate must fall by this amount during the reaction. Thus for experiment 1 this

quantity is $10(1 - 63/120)$, or 4.75 mM . In calculating K_m it is necessary therefore to take into account that the citrate concentration fell from 40 to 35.25 mM , and this can be done with sufficient accuracy by substituting for the initial concentration the average concentration during the experiment, *i.e.* 37.6 instead of 40 . Then the corrected value of K_m is $37.6 \left(\frac{V_{\max}}{V} - 1 \right)$ and this is $37.6 \left(\frac{1}{0.21} - 1 \right)$, or 141 , instead of 150 , which is the uncorrected figure. In Table V the corrected Michaelis constants for each experiment are tabulated.

Table V.

The figures for the Michaelis constant corrected for the fall of citrate concentration during the reaction.

Exp. No.	1	2	3	4	5	6	7	8	9	10
K_m (corr.)	142	281	137	130	137	65	71	70	115	78
Mean value K_m at 25° 165 mM .										
" " " 35° 80 "										

From these figures the heat of formation of the enzyme-substrate compound is calculated by substitution in the integrated form of the reaction isochore. The expression

$$\frac{d(\log_e K)}{dT} = - \frac{Q}{RT^2}$$

can be integrated on the assumption that Q is constant, giving

$$\log_e \frac{K_2}{K_1} = - \frac{Q(T_2 - T_1)}{RT_1 T_2},$$

where K_2 and K_1 are the equilibrium constants at T_2 and T_1 respectively. The heat of reaction is generally assumed to be constant over a small temperature range such as 10° , so by substitution, since the equilibrium constant of the forward reaction $E + S \rightarrow ES$ is the reciprocal of the Michaelis constant:

$$2.3 \log_{10} \frac{K_m^{25^\circ}}{K_m^{35^\circ}} = \frac{Q}{1.99} \times \frac{(308 - 298)}{298 \times 308},$$

$$\text{therefore} \quad 2.3 \log \left(\frac{165}{80} \right) = \frac{Q}{1.99} \times \frac{10}{298 \times 308}$$

$$\text{and} \quad Q = + 2.3 \times 0.3144 \times 1.99 \times \frac{298 \times 308}{10} = + 13460 \text{ cal.}$$

The heat liberated by the combination of citric acid dehydrogenase and citric acid is 13460 cal. per g. molecule of citric acid.

(2) *The heat of activation of the enzyme-substrate compound.* The mean of the three figures obtained for the ratio of maximum reaction velocity at 35° to the maximum velocity at 25° is 1.65 , and by substitution in the expression

$$E = \frac{RT_1 T_2}{T_2 - T_1} \log_e \frac{k_2}{k_1},$$

the energy of activation is found to be 9139 cal. per g. molecule.

DISCUSSION.

The observations described above lead definitely to the conclusion that when the citric acid dehydrogenase combines with its substrate a large amount of heat is set free, and although the figure given is not accurate, it can be said with confidence that the heat of formation of the enzyme-substrate compound is about 12,000 or 15,000 cal. per g. molecule. This is a fairly large figure, and larger indeed than the heat of activation of the enzyme-substrate compound, which is about 9000 cal. per g. molecule. As far as the writer is aware the only other enzyme for which both the corresponding energy values have been determined is yeast saccharase. Euler and Laurin [1920] measured the heat of formation of the saccharase-sucrose compound and concluded that it was about 2000 cal. per g. molecule. On the other hand, Nelson and Bloomfield [1924] in the course of a very careful study of the kinetics of this enzyme found that the heat of formation of the enzyme-substrate compound was zero within the limits of experimental error. The heat of activation of the saccharase-sucrose compound was found by them to be 8400 cal. per g. molecule at 30°, in agreement with the figures obtained by Euler and Laurin and other workers.

It is evident that although the energy of activation of the enzyme-substrate compound is of the same order for yeast saccharase and for citric acid dehydrogenase at 30°, the heat of formation of the compound at the same temperature is quite large for the dehydrogenase and zero for the saccharase. Whether this distinction is a general one between oxidising and hydrolysing enzymes cannot be decided until similar data are available for many other enzymes of both classes, but the possibility must be born in mind. If it holds, it will take its place with the generalisations that hydrolyses proceed with much smaller heats of reaction than oxidations, and that the Michaelis constants of oxidising enzymes are much smaller than those of hydrolysing enzymes.

The fact that in the case of the citric acid dehydrogenase the heat of formation of the enzyme-substrate compound is greater than the energy of activation of the same compound, suggests that the action of the enzyme may be pictured in terms of the energy exchanges as follows: the enzyme molecule unites with a molecule of substrate and heat energy is thereby released which can immediately be absorbed by the enzyme-substrate compound. This absorption of energy by the compound serves to activate it and it then passes from the activated state to the final state of enzyme molecule + molecules of the product or products. Such a sequence of energy exchanges could not however apply when an enzyme-substrate compound has a heat of formation less than its energy of activation, as is the case with saccharase.

SUMMARY.

The Michaelis constant of the citric acid dehydrogenase of cucumber seeds has been measured at 25° and at 35°. At 25° it is $1.65 \times 10^{-4} M$, and at 35° it is $8 \times 10^{-5} M$. Thus the temperature coefficient $Q_{10} = 0.48$ at 30° and

the heat of formation of the citric dehydrogenase-citric acid compound as calculated from the temperature coefficient is 13460 cal. per g. molecule.

The temperature coefficient of the velocity of the reaction catalysed by the dehydrogenase has also been measured and $Q_{10} = 1.65$ at 30° . From this the calculated energy of activation of the citric dehydrogenase-citric acid compound is 8790 cal. per g. molecule.

These figures are contrasted with the corresponding ones for yeast saccharase and the significance of the contrast is discussed briefly.

My thanks are due to Professor T. Thunberg for advice concerning the use of the dehydrogenase, and to Professor J. B. S. Haldane for many valuable suggestions. I wish also to thank Sir F. G. Hopkins for much help and encouragement.

REFERENCES.

- Bernheim (1928). *Biochem. J.* **22**, 1178.
Euler and Laurin (1920). *Z. physiol. Chem.* **110**, 55.
Haldane (1930). *Enzymes* (Longmans Green and Co., London).
Michaelis and Menten (1913). *Biochem. Z.* **49**, 333.
Nelson and Bloomfield (1924). *J. Amer. Chem. Soc.* **46**, 1025.
Quastel (1926). *Biochem. J.* **20**, 166.
--- and Wooldridge (1927). *Biochem. J.* **21**, 1224.
- - - (1928). *Biochem. J.* **22**, 689.
Scherstén (1929). *Skand. Arch. Physiol.* **58**, 90.
Thunberg (1928). *Die Methodik der Fermente* (Oppenheimer-Pincussen, Thieme, Leipzig), pp 1118-1134.
---- (1929). *Biochem. Z.* **206**, 109.

XXV. THE AMINO-ACIDS OF GLUTENIN.

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THE necessity for investigating new methods of protein analysis requires no emphasis. While the separation of the bases by the method of Kossel and Patten [1903], especially as recently modified by Vickery and Leavenworth [1928], and the precipitation of the dicarboxylic acids by calcium hydroxide and alcohol [Foreman, 1914] lead to nearly quantitative results, the isolation of the monoamino- and non-amino-monocarboxylic acids by the usual ester method leaves much to be desired both from the point of view of purity of products and of yield. The different modifications of the ester method that have been devised have been successful in overcoming certain of its shortcomings. Preliminary extraction of the amino-acids as recommended by Dakin [1918] and the use of their lead salts for esterification adopted by Foreman [1919] lead to anhydrous preparations of the monoamino-acids which are therefore more readily esterified.

However in any separation of the amino-acids based on the fractionation of their esters certain serious drawbacks inherent in the method are bound to remain, viz. (i) loss of esters due to volatilisation and to decomposition during distillation, (ii) the difficulty of separating closely related substances which may appear in the same fraction of the distillate, and (iii) the presence of unmanageable syrups which hinder purification and crystallisation. In this connection Osborne's observations [1910] on the separation of a synthetic mixture of pure amino-acids—in which the recovery was only 72.3 %—are of significance. It is clear then that a successful method for the separation of amino-acids which does not involve the preparation and distillation of the esters would form a valuable contribution to the study of the amino-acid composition of the proteins.

Some of the results of a series of investigations carried out in this laboratory under the direction of the late Professor Schryver with the object of separating the amino-acids by taking advantage of the differences in the solubilities of their copper salts have already been published by Town [1928] and by Brazier [1930]. In the present paper the results of experiments on the separation of the copper salts of the amino-acids from wheat glutenin are described.

It has been possible to obtain higher yields of alanine, valine, proline and aspartic acid than those previously obtained by the ester method. Since the

completion of this work, the publication of which has for various reasons been considerably delayed, two of the increased values have been confirmed in other laboratories. Spörer and Kapfhammer [1930], using Reinecke salt for the precipitation of proline, have obtained a value (5.98 %) practically identical with that found in this investigation (6.15 %). Jones and Moeller [1928], who have used essentially the same procedure as that described in this paper for the isolation of the dicarboxylic acids, have found 2 % aspartic acid in close agreement with the value (1.85 %) now reported. Phenylalanine and leucine are found in the same fraction in both the copper salt and the ester method, and they have not been satisfactorily separated. The actual yields of these two substances are lower than those found by Osborne, but the use of an indirect method involving the oxidation of phenylalanine to benzoic acid has raised the value of phenylalanine to 2.75 % (ester method, 1.97 %).

Apart from the higher yields of certain individual amino-acids it has been possible to trace a large proportion of the nitrogen not accounted for in the products isolated. The nitrogen entirely unaccounted for amounts to only about 8 % of the total, a loss which can hardly be avoided considering the nature of the processes involved. By checking the amount of nitrogen lost by adsorption on various precipitates it has been found possible to gain some idea of the deficiencies that fall upon particular amino-acids. By this means also it has been possible to devise modifications of the method so as to obviate such losses in future hydrolyses.

Separation of the amino-acids is effected by taking advantage of the difference in solubility of their copper salts in water and in methyl alcohol. The extraction, under carefully regulated conditions, of the dry copper salts of the mixed amino-acids by these two solvents in succession gives three fractions.

(i) Copper salts soluble in methyl alcohol: *valine* and *proline*.

(ii) Copper salts insoluble in methyl alcohol but soluble in water under the conditions of the experiment: *glycine*, *alanine*, *aspartic acid*, *glutamic acid*, *arginine*, *histidine*, *lysine* and some *tyrosine*.

(iii) Copper salts insoluble in methyl alcohol and in water: *leucine*, *phenylalanine* and *aspartic acid*.

In each of the above fractions, especially (i) and (ii), a certain amount of unidentified matter was also present. The amino-acids in each fraction have been separated by taking advantage of the difference in solubility either of the acids themselves or of their derivatives in suitable solvents.

EXPERIMENTAL.

Preparation of the glutenin. Gluten, obtained by thorough washing of wheat flour, was repeatedly extracted with 73 % alcohol to remove the gliadin. The residue was dried at room temperature, ground to a fine powder and exhaustively extracted with alcohol. Excess of alcohol was removed at room

temperature with the help of a fan and the residue was dissolved in 0.2 % sodium hydroxide. The solution was filtered clear and precipitated by the addition of dilute acetic acid. The product was purified by reprecipitation from sodium hydroxide. It was then washed free from sodium acetate with dilute alcohol and finally dehydrated with acetone. The glutenin thus prepared contained 8.36 % water and 0.44 % ash, and, ash- and moisture-free, 17.02 % nitrogen.

Hydrolysis. 250 g. of glutenin equivalent to 228 g. of ash- and moisture-free protein and to 38.81 g. of nitrogen were hydrolysed by boiling with four times the weight of 25 % sulphuric acid under a reflux condenser for 24 hours. The cooled solution was diluted to 4 litres and the humin was filtered off, washed thoroughly with hot water and analysed for nitrogen. Ammonia was determined in a small aliquot by aeration. Most of the sulphuric acid was now precipitated by the addition of saturated baryta to the hydrolysate. The barium sulphate was repeatedly washed with hot water, then dried at 110° and washed again after powdering. A nitrogen determination on the barium sulphate showed that it retained 6.47 % of the total nitrogen of the protein. The filtrate and washings from the barium sulphate were concentrated to about 2 litres and the sulphuric acid was quantitatively removed. On further concentration to 750 cc. a precipitate of crude tyrosine separated which gave, after recrystallisation, 6.84 g. of the pure amino-acid.

Preparation of the dry copper salts. The filtrate from the tyrosine was heated on a water-bath in a large porcelain dish and treated with copper carbonate in small portions at a time. After the addition of a fairly large excess of copper carbonate the solution was evaporated to a thick syrup which was left on the water-bath for some hours. It was then taken up again in water and filtered from the excess of copper carbonate, and the latter was washed thoroughly with boiling water. The filtrate and washings were once more evaporated to a syrup with excess of copper carbonate to ensure complete conversion into the copper salts. After again taking up in water and filtering, the solution was evaporated to a thick syrup, allowed to cool and then stirred up with a liberal amount of acetone. After standing for a short time the acetone was poured off and replaced by a fresh quantity. On repeating this process three to four times a dark blue friable mass was obtained which was easily ground to a fine powder. This was filtered with suction and freed from acetone in a vacuum desiccator over sulphuric acid. It was then dried thoroughly at 110° for 24 hours. If the syrup is evaporated too far before granulation with acetone or if an appreciable amount of acetone is present in the mixture when placed in the drying oven hard cakes are obtained which are difficult to break up and impossible to dehydrate completely.

The thoroughly dried copper salts weighed 279 g. and contained 26.53 g. of nitrogen.

Fractionation of the copper salts.

(a) *Extraction with methyl alcohol.* The thoroughly dried and finely ground copper salts were shaken with three times their volume of dry methyl alcohol (freshly distilled from sodium) on a shaking machine. After about 2 hours the blue solution was filtered and fresh methyl alcohol was added to the residue. Seven extractions were usually sufficient to remove all soluble material. The combined extracts were filtered at the pump, evaporated to a syrup and the residue was granulated with acetone. After drying for one hour at 100° and taking up once more in methyl alcohol a small residue was usually obtained which was put back into the main fraction of copper salts. The solution when evaporated gave a residue completely soluble in methyl alcohol.

(b) *Extraction with water.* The residual copper salts were extracted four times successively with large volumes of water at 60°. The aqueous solutions were evaporated and the residue was dehydrated with acetone. The extraction was repeated on this residue, using very large volumes of warm water. Any insoluble residue was added to Fraction III consisting of the copper salts insoluble in both methyl alcohol and water. The three fractions were decomposed with hydrogen sulphide, I and II in aqueous solution, III in dilute sulphuric acid. The copper sulphide obtained in each case was very thoroughly washed and after drying was analysed for nitrogen. The filtrate and washings in each fraction were evaporated *in vacuo* to remove hydrogen sulphide and the nitrogen was determined in an aliquot in each case.

Fraction I. Amino-acids from copper salts soluble in methyl alcohol.

On evaporating the aqueous solution a pale brown sticky mass was obtained. This was thoroughly dehydrated by repeated addition of absolute alcohol and evaporation *in vacuo*. It was next exhaustively extracted with hot absolute alcohol. The insoluble residue was white and friable. The alcoholic extracts were evaporated and the residue was re-extracted with alcohol; a small amount of undissolved material was added to the main insoluble portion. This operation was repeated until the material obtained on evaporation was completely soluble in absolute alcohol.

Valine. The residues insoluble in ethyl alcohol were combined and re-converted into their copper salts. The material recovered by decomposition of the latter was entirely insoluble in alcohol. When recrystallised from water it had m.p. 313–315°, N 11.92 %, and was therefore valine. Yield 2.33 g. No alcohol-soluble zinc salt suggestive of hydroxyvaline [Schryver and Buston, 1926] could be obtained.

Proline. The material completely soluble in alcohol was recovered by evaporation and taken up in water. The solution was analysed for non-amino-nitrogen, diluted until the concentration of the latter was about 1 g. per 100 cc., brought to the boil and treated with a hot solution containing sufficient picric acid to combine with the non-amino-nitrogen present [Town,

1928]. After evaporating and cooling proline picrate crystallised out in the form of golden yellow needles. On further evaporation of the mother-liquor a second small crop was obtained. The proline picrate was washed at the pump with cold water and, after drying in the desiccator, was extracted with ether to remove excess of picric acid as well as another picrate found to be soluble in ether (see later).

The aqueous and ethereal filtrates from the proline picrate were freed from picric acid by means of sulphuric acid and ether. After the quantitative removal of sulphuric acid the solution was evaporated to a syrup, taken up in 95 % alcohol and treated with a saturated solution of cadmium chloride [Kapfhammer and Eck, 1927] which was cautiously added drop by drop until no further precipitation took place. The precipitate was filtered off and washed with cold 95 % alcohol until free from chloride, re-dissolved in water and freed from cadmium chloride (chloride precipitated by silver sulphate, and silver and cadmium by hydrogen sulphide). After the quantitative removal of sulphuric acid the solution was once more treated with picric acid. The combined proline picrate precipitates were recrystallised from water, when 42 g. of picrate melting at 148° were obtained corresponding to 14.02 g. of proline.

The mother-liquors from which proline had been recrystallised as the picrate and the residual proline had been precipitated as the cadmium chloride compound were freed quantitatively from all reagents and the aqueous solution was evaporated to a syrup. It contained 5.91 % of the total nitrogen. No crystallisation took place on long standing in the desiccator, and it was found impossible to obtain it in a dry state. No definite compound was isolated from this mixture, but it could be separated into two fractions, one forming a picrate soluble in ether, the other not giving a picrate.

Fraction II. Amino-acids from copper salts insoluble in methyl alcohol, but soluble in water.

Tyrosine. On evaporation of the amino-acid solution to about 600 cc. a small amount of tyrosine crystallised out. The weight of pure recrystallised material was 2.76 g., making with that previously isolated 9.6 g. of tyrosine in all.

Aspartic and glutamic acids. The solution was treated according to Kingston and Schryver's modification [1924] of Foreman's [1914] method for the isolation of the dicarboxylic acids. It was found that by adding baryta in a thin cream in fair excess and keeping the mixture vigorously stirred during the slow and gradual addition of alcohol the formation of a sticky precipitate could be avoided. The barium salts were re-dissolved in water and again precipitated with alcohol in the presence of excess of baryta. The filtrate and washings from this second precipitation were not added to the main filtrate but were examined separately. The precipitate was dissolved in water and the barium was removed quantitatively. The crystals obtained from this

solution on evaporation, though possessing the characteristic form of glutamic acid, were found to be impure and therefore they were fractionated by the procedure adopted by Dakin [1919]. Glutamic acid was separated in three successive crops as the hydrochloride, 72.65 g. of recrystallised hydrochloride, corresponding to 58.14 g. of glutamic acid, being obtained.

From the mother-liquors after removal of glutamic acid aspartic acid was separated as the lead salt [Dakin, 1919]. The yield of pure recrystallised aspartic acid was 0.52 g.

By treatment of the filtrate from the lead aspartate with sodium hydroxide and silver nitrate the silver salt of hydroxyglutamic acid was precipitated. It was not however found possible to obtain crystals of hydroxyglutamic acid even after long standing in the vacuum desiccator. The hard, dry mass thus obtained weighed 2.3 g. corresponding to 1 % of the weight of glutenin. It contained 0.54 % of ash. (N: found, 8.40 %; calc., 8.59 %.)

Glycine. The filtrate and washings obtained from the second precipitation of the barium dicarboxylates were freed from alcohol and barium. The solution gave a negative reaction with Millon's reagent showing the absence of tyrosine. A small precipitate was obtained with phosphotungstic acid. It was filtered off, decomposed and the solution so obtained was added to the main filtrate from the dicarboxylic acid precipitation. The filtrate was freed from phosphotungstic acid and the solution was evaporated to dryness. The residue obtained consisted of glycine mixed with a certain amount of inorganic impurity. By recrystallisation 0.6 g. of pure glycine was obtained. (N: found, 18.57 %; calc., 18.67 %.) It was further identified by conversion into the picrate m.p. 189°.

Removal of the bases. In the main filtrate from the dicarboxylic acid precipitate arginine and histidine were precipitated as their silver salts by the method of Vickery and Leavenworth [1928]. The nitrogen in the solution obtained by decomposing the silver precipitate was 11.6 % of the total in good agreement with Osborne's [1910] value of 11.39 %. From the solution, after removal of arginine and histidine, lysine was separated in the usual way as picrate. After its removal the residual solution still contained 1 g. of nitrogen, which was not identified.

Alanine and glycine. The solution with the washings from the phosphotungstic acid precipitation above was freed from phosphotungstic and sulphuric acids and evaporated to 500 cc., and the amino-acids were converted into their barium carbamates by the method of Kingston and Schryver [1924]. The precipitate was dissolved in water at room temperature and freed from barium. On evaporation glycine (1.13 g.) crystallised out, making, with the amount already isolated, a total weight of 1.73 g.

The washings containing the soluble barium carbamate were boiled and the precipitated barium carbonate was filtered off. The filtrate, on evaporation, gave nearly pure alanine. The yield after recrystallisation from aqueous alcohol was 14.04 g. (N: found, 15.83 %; calc., 15.73 %.) In view of the difficulty of effecting a complete separation of alanine and glycine, either by

the ester method or by the formation of their calcium chloride compounds [Pfeiffer and Wittka, 1915] it may be pointed out that the procedure described above gives a very clean and satisfactory separation of these two substances.

Fraction III. Amino-acids from copper salts insoluble in methyl alcohol and water.

The copper salts in this fraction were brought into solution by suspending them in water and adding small quantities of sulphuric acid from time to time as necessary, and were decomposed as before.

The alcohol-insoluble barium salts from this fraction yielded, when decomposed, *aspartic acid*. (Yield after recrystallisation from water 3.7 g., making, in all, 4.22 g. or 1.85 % of the weight of glutenin.)

Leucine and phenylalanine. The main filtrate from the barium aspartate was freed from alcohol and barium and evaporated to a small volume on the water-bath. Most of the material crystallised out in flakes which were filtered off and washed with cold water. On evaporating the mother-liquor and washings a small second crop of crystals was obtained. The dry crystals contained 10.03 % N. The mother-liquor, on evaporation to dryness, gave a crystalline powder, the nitrogen content of which (10.01 %) showed it to be of similar composition to the earlier crops of crystals. The method of fractionation employed suggested that this material was a mixture of leucine and phenylalanine. Separation of the two amino-acids however was only partially successful. By repeated fractional crystallisation 8.5 g. of nearly pure leucine was obtained. (m.p. 280–285°. N: found, 10.4 %; calc., 10.69 %.) The residual mixture (12 g. containing 9.76 % nitrogen) was converted into the zinc salt [Brazier, 1930]. From the water-soluble portion of the dried salts, after removal of zinc, 3.45 g. of crude phenylalanine was obtained. (N: found, 8.86 %; calc., 8.49 %.)

The residue insoluble in cold water was made alkaline with a little barium hydroxide and decomposed with hydrogen sulphide. On evaporating the solution 8.5 g. of material having 10.13 % N crystallised out. As it was found impossible to effect any further separation either by crystallisation or by the use of the zinc salts the phenylalanine content was determined indirectly by oxidation to benzoic acid [Kollmann, 1928]. The mixture was oxidised by boiling for 6 hours with Beckmann's solution; the benzoic acid, together with the fatty acids formed, were extracted in a continuous extractor with ether, and after evaporation of the ether the benzoic acid was separated by washing the residue with water previously saturated with benzoic acid. The benzoic acid was dried to constant weight at 60°. Duplicate experiments gave the following results: 1.505 g. of the mixture yielded 0.3476 g. of benzoic acid, equivalent to 23.33 % of phenylalanine; 1 g. gave 0.1645 g. of benzoic acid equivalent to 23.66 % of phenylalanine. 0.5 g. of pure phenylalanine gave 0.3476 g. of benzoic acid corresponding to a yield of 94 %. The mixture thus contained 23.5 % phenylalanine and 76.5 % leucine. Calculated from the nitrogen

content the figures are 25.5 % and 74.5 % respectively. As the discrepancies are within the limits of error of the oxidation method it seems legitimate to assume that the mixture consisted only of leucine and phenylalanine and to adopt the latter of the above sets of figures as representing its composition. On this assumption the gross value for phenylalanine is 2.75 % and for leucine 6.3 % of the glutenin.

The following tables summarise the results obtained. Table I gives the distribution of nitrogen in the main fractions at the stage prior to the liberation of the amino-acids from their copper salts. Table II gives the percentages of the different products isolated, both in terms of total nitrogen and of total weight of protein taken, together with the values obtained by previous workers for comparison. Column 2 of Table II gives an indication of the losses encountered; those in precipitates are in some cases ascribed to particular amino-acids. It is seen, for example, that the value for glycine is at least 20 %, for alanine 5 % and for aspartic acid 10 % too low. By combining the highest values from the present analysis with those obtained by previous workers 70.98 % of the protein is now accounted for in the products of hydrolysis. In the case of the monoamino-acids, with which this method is chiefly concerned, the value 22.14 % given by the ester method has been raised to 27.34 %.

Table I. *Distribution of nitrogen in different fractions.*

	Nitrogen in g.	Nitrogen %
Humus	0.45	1.17
In main barium sulphate precipitate	2.51	6.46
In solution	35.06	90.36
Loss	0.79	2.01
Total	38.81	100.0
In tyrosine isolated before forming copper salts ...	0.53	1.36
In ammonia	7.41	19.10
In excess copper carbonate	0.26	0.67
In copper salts	26.53	68.38
Loss	0.33	0.85
Total	35.06	90.36
In Fraction I of the copper salts	4.64	11.96
In Fraction II	17.75	45.74
In Fraction III	3.17	8.17
Loss	0.97	2.51
Total	26.53	68.38

DISCUSSION.

The following advantages can be claimed for the method of separation described.

1. By avoiding the losses inherent in the ester method higher yields of most of the monoamino-acids have been obtained, notably in the case of proline, valine, alanine and phenylalanine.

Table II.

	% of total nitrogen. Copper salt method		% of weight of protein	
	In products isolated	In precipitates and unidentified syrups	Copper salt method	Other methods
Humin	—	1.17	—	—
Main barium sulphate precipitate ...	—	6.46	—	—
<i>Ammonia</i>	19.1	—	3.97	4.01 ¹
Excess copper carbonate... ..	—	0.67	—	—
<i>Tyrosine</i> (both fractions)... ..	1.91	—	4.20	4.25 ¹
Fraction I:				
Copper sulphide	—	0.53	—	—
<i>Valine</i>	0.72	—	1.02	0.21
<i>Proline</i>	4.39	—	6.15	5.98 ²
Unidentified syrup	—	5.91	—	—
Fraction II:				
Copper sulphide	—	1.60	—	—
Barium sulphate from first precipitation	—	0.22	—	—
<i>Glutamic acid</i>	14.78	—	26.49	25.7 ³
<i>Hydroxyglutamic acid</i>	0.5	—	1.00	1.8 ⁴
Lead hydroxide and sulphate	—	0.07	—	—
<i>Arginine and histidine</i>	11.60	—	—	—
Silver and barium precipitates	—	0.25	—	—
Lysine and unidentified matter	—	6.42	—	—
Barium phosphotungstate	—	0.20	—	—
<i>Glycine</i> (from Fractions I and II) ...	0.83	—	0.76	0.89 ¹
Barium sulphate from carbamate ...	—	0.19	—	—
<i>Alanine</i>	5.68	—	6.16	4.65 ¹
Barium sulphate	—	0.29	—	—
Fraction III:				
Copper sulphide	—	1.05	—	—
Barium sulphate	—	0.09	—	—
<i>Aspartic acid</i>	1.15	—	1.85	2.0 ³
<i>Leucine</i>	3.96	—	6.3	5.95 ¹
<i>Phenylalanine</i>	1.37	—	2.75	1.97 ¹

¹ Osborne and Clapp [1906].³ Jones and Moeller [1928].² Spörer and Kapfhammer [1930].⁴ Dakin [1919].

2. The indeterminate losses of the ester method are replaced by definite losses in precipitates which can be readily determined. Thus some idea is gained of the deficiencies falling on individual amino-acids.

3. The fractionation of the copper salts gives rise to groups of amino-acids which are more amenable to separation than are the fractions obtained by conversion into the esters. Thus alanine, valine and the leucines, which in the ester method form one group extremely difficult to separate, are here obtained in three fractions.

4. The amino-acids set free from the copper salts are, as a rule, free from contaminating syrupy material and therefore readily crystallisable.

On the other hand, the procedure described here, though adopted after

many preliminary separations on rather different lines, still suffers from the following drawbacks.

(a) The considerable loss of the hydrolysis products in the first barium sulphate precipitate.

(b) The difficulty of separating the copper salts of aspartic and glutamic acids in the presence of large amounts of the latter.

(c) The inability to isolate serine and cystine. In one preliminary experiment the dicarboxylic acids and the diamino-acids were first removed, the former as the barium salts, the latter by means of phosphotungstic acid, prior to conversion into copper salts. This procedure was abandoned on the ground that the barium dicarboxylates carried traces of other amino-acids with them and the basic phosphotungstates were not completely insoluble. Later experience has however shown that the preliminary removal of these two groups of amino-acids would considerably simplify the fractionation of the copper salts of the monoamino-acids and in future work this modification is recommended.

SUMMARY.

1. The hydrolysis products of glutenin have been fractionated by means of their copper salts.
2. Higher yields of many of the monoamino-acids have been obtained.
3. Possible improvements in the method are indicated.

Grateful acknowledgment is made to the late Professor S. B. Schryver, at whose suggestion this work was undertaken.

REFERENCES.

- Brazier (1930). *Biochem. J.* **24**, 1191.
Dakin (1918). *Biochem. J.* **12**, 290.
—— (1919). *Biochem. J.* **13**, 398.
Foreman (1914). *Biochem. J.* **8**, 463.
—— (1919). *Biochem. J.* **13**, 378.
Jones and Moeller (1928). *J. Biol. Chem.* **79**, 429.
Kapfhammer and Eck (1927). *Z. physiol. Chem.* **170**, 294.
Kingston and Schryver (1924). *Biochem. J.* **18**, 1070.
Kollmann (1928). *Biochem. Z.* **194**, 1.
Kossel and Patten (1903). *Z. physiol. Chem.* **38**, 39.
Osborne (1910). *Amer. J. Physiol.* **26**, 305.
—— and Clapp (1906). *Amer. J. Physiol.* **17**, 231.
Pfeiffer and Wittka (1915). *Ber. deutsch. chem. Ges.* **48**, 1041.
Schryver and Buston (1926). *Proc. Roy. Soc. Lond. B* **99**, 476.
Spörer and Kapfhammer (1930). *Z. physiol. Chem.* **187**, 84.
Town (1928). *Biochem. J.* **22**, 1083.
Vickery and Leavenworth (1928). *J. Biol. Chem.* **76**, 377, 707.

XXVI. STUDIES ON THE GUMS.

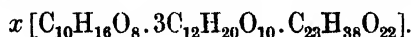
II. TRAGACANTHIN—THE SOLUBLE CONSTITUENT OF GUM TRAGACANTH.

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THE literature on the subject of gum tragacanth, like that of the gums in general, is fragmentary and confused. The first work of value is that of Ogle [1900] who stated that this gum is not a simple substance, but contains 36 % of a water-soluble carbohydrate constituent and 42 % of an insoluble one, which swells up in water. The soluble constituent was stated to give a product on precipitation with alcohol, different from arabin (acid gum arabic). Widstoe and Tollens [1900] treated gum tragacanth as a simple substance, from which they obtained on hydrolysis the sugars, fucose and arabinose, and from another sample, xylose and arabinose. In the same year Hilger and Dreyfus [1900] stated that this gum contained 15-22 % galactose, estimated as mucic acid, and 30-42 % arabinose. Tollens [1901], as a result of ultimate analyses, formed the opinion that this gum contained carboxyl groups, a view contrary to that held by Hilger and Dreyfus. O'Sullivan [1901] gave an account of some very detailed work on the composition of this gum. He opens with the statement that gum tragacanth had been held to be a mixture of "cellulose, starch, bassorin, a gum-like arabin, a little nitrogenous matter, sugar, and ash." The soluble constituent "gum-like arabin" he concludes to be a mixture of poly-arabinon-trigalactan-geddic acids and gives for it the formula



More recently von Fellenberg [1914] states that whereas the bassorin or insoluble portion is methoxylated to the extent of 5.38 %, the water-soluble constituent has no ester group of this type.

It seems, strangely enough, that a name has never been given to this constituent of gum tragacanth soluble in water, and it is proposed to term it tragacanthin.

EXPERIMENTAL.

Gum tragacanth on addition of water swells enormously, since the water-insoluble form, bassorin, constituting some 60-70 % of the gum, gives a very bulky jelly. O'Sullivan [1901] stated that he effected a separation of the soluble gum from the bassorin by means of fractional precipitation with alcohol. A solution of gum tragacanth in water was concentrated until a gelatinous scum began to form on the surface of the liquid. Dilute alcohol was added

gradually until a bulky and curdy precipitate came down, which was stated to be bassorin. From the alcoholic filtrate, on evaporation and addition of strong alcohol, a further precipitate was obtained, claimed to be the tragacanthin. This he fractionated several times, but nevertheless it cannot be held to be an ideal method of separation. Some more reliable method was sought for in this work, and many methods both physical and chemical were tried. For a time a stream-line filter works admirably with a suitable grade of paper, but unless it is very large it chokes before any considerable quantity has filtered. Finally, filtration on very large fluted filter-papers was adopted. This was an extremely laborious method since filtration was slow, and since a concentration of the gum higher than about 0.1 % could not be employed. The filter-papers have frequently to be changed; but nevertheless such a method makes it absolutely certain that the product obtained is tragacanthin only. The very dilute filtrate was concentrated under reduced pressure to a small bulk, and several volumes of 95 % alcohol were added, together with a small quantity of hydrochloric acid. A stringy precipitate formed, not unlike many pectin preparations in appearance. This was filtered off, dissolved in water, and again precipitated with acid alcohol. After several such reprecipitations the product was dried in absolute alcohol and finally in a desiccator over phosphorus pentoxide.

A satisfactory separation was also effected in the following manner. The solution of the gum was first made alkaline and then just slightly acid by the addition of acetic acid, whereupon the physical condition of the bassorin is somewhat altered, and it appears as large gelatinous flocks. These were removed by running the liquid through a super-centrifuge. The effluent from the centrifuge was allowed to stand and the clear supernatant liquid finally filtered through filter-paper as before. The final product in each case was a fine white powder very readily soluble in cold water.

A portion was hydrolysed by boiling for one hour with sulphuric acid, and the solution examined for sugars after the removal of the acid by baryta. Three volumes of hot alcohol were added to the hydrolysate to remove incompletely hydrolysed gum and the filtrate concentrated under reduced pressure to a thin syrup which was then utilised for the tests. Contrary to the observation of O'Sullivan [1901] galactose was not found, for only a trace of mucic acid remained on oxidation with nitric acid. The only sugar that could be detected was arabinose, which was present in some quantity. This was identified by means of its diphenylhydrazone.

The yield of carbon dioxide as a measure of uronic acid content was determined in the usual way.

<i>Tragacanthin.</i> Sample I	Ash	0.87 %
Yield of CO ₂ on ash-free basis	(i)	12.65 %
				(ii)	12.75 %

Mean value 12.70 % corresponding to 50.8 % uronic acid anhydride.

It is not possible to be definite as to the nature of the uronic acid, though there is some evidence for supposing that it is galacturonic.

The yield of furfuraldehyde was next determined so that a figure could be obtained by calculation for the pentose present in the molecule.

Yield of furfuraldehyde on ash-free basis...	(i)	31.26 %
	(ii)	31.42 %
Mean value		31.34 %

Since uronic acids themselves yield 16.66 % of furfuraldehyde, the 50.8 % uronic acid anhydride present in the molecule is responsible for the yield of 8.48 % furfuraldehyde, leaving the balance 22.88 % as due to pentose. This, calculated as anhydroarabinose, is 43.12 %.

Since no sugar other than arabinose could be detected in the hydrolysis liquid, it seems likely that tragacanthin consists solely of uronic acid and arabinose, though it is only possible to account for 94 % of the molecule in this way. There is a great divergence between this conclusion and that of O'Sullivan [1901], who considered tragacanthin to be a complex poly-arabinon-trigalactan-geddlic acid, yielding on hydrolysis, arabinose, galactose, and geddic acid, an isomer of arabic acid also obtained by him from certain constituents of gedda gum. It seems likely that the preparation obtained by O'Sullivan contained some bassorin, which would account for the presence of galactan units.

To throw further light on the constitution of this gum several hydrolyses were carried out, and an examination of the hydrolysis products was undertaken. A portion of tragacanthin was dissolved in 3 % sulphuric acid and boiled under reflux for the period stated. It was then nearly neutralised with barium carbonate while still hot, filtered and poured into hot alcohol. A precipitate rapidly settled out and the supernatant liquid was poured off before it cooled. The precipitate was redissolved, filtered and reprecipitated with hot alcohol as before. This process was repeated several times in each case to ensure the removal of all free sugar. Finally it was dried with absolute alcohol and over phosphorus pentoxide.

The following analytical figures were obtained for the various preparations:

Table I. *Hydrolysis of tragacanthin with 3 % sulphuric acid.*

Preparation	Tragacanthin sample I %	30 minutes' hydrolysis %	60 minutes' hydrolysis %	90 minutes' hydrolysis %
Ash	0.87	1.04	2.87	3.70
CO ₂ yield	12.70	18.83	19.27	20.28
Furfuraldehyde yield	31.34	23.76	23.18	22.20
Uronic acid anhydride	50.80	75.32	77.08	81.12
Furfuraldehyde from uronic acid	8.46	12.55	12.84	13.50
Furfuraldehyde from pentose	22.88	11.21	10.34	8.70
Anhydroarabinose	43.10	20.89	19.27	16.25

The composition of the hydrolysis products is illustrated in Fig. 1. It will be noted that during the initial period of hydrolysis the arabinose content

diminishes rapidly, and the uronic acid increases proportionately. After 30 minutes, however, very little more arabinose is removed during a further period of 30 minutes, and even up to 90 minutes the product remains of much the same order. After this period hydrolysis soon becomes complete. These

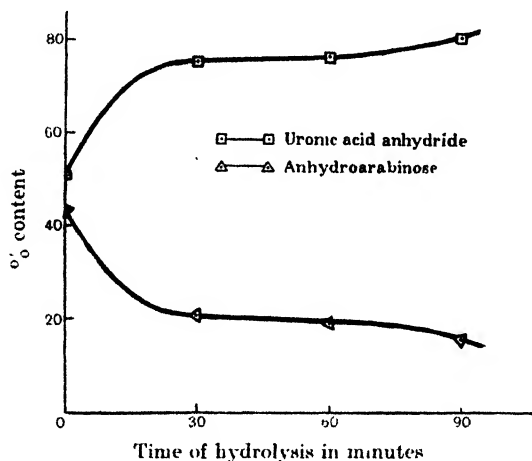
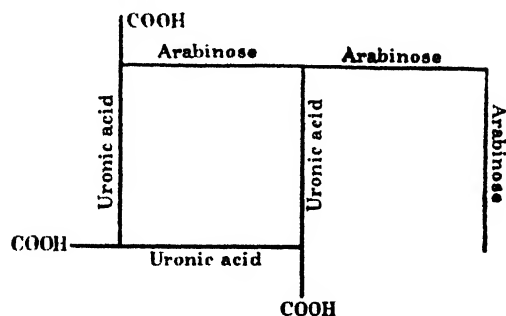


Fig. 1. Hydrolysis of tragacanthin with 3% H_2SO_4 .

results throw a considerable amount of light on the constitution of the tragacanthin molecule. It is clear that the uronic acid and a portion of the arabinose form a more resistant nucleus acid, probably linked in the form of a ring. Attached to this by a glucosidic linkage must be the remaining arabinose, which is thus easily removed on hydrolysis. A simple arrangement agreeing fairly closely with the figures experimentally obtained is a nucleus acid consisting of three molecules of uronic acid linked with one of arabinose to form a four-membered ring, attached to which is a side chain of two molecules of arabinose, as illustrated diagrammatically below.



Such a nucleus would have a composition of approximately 20.0% anhydroarabinose and 80.0% uronic acid anhydride, which is not dissimilar from that corresponding with the figures obtained after hydrolysis for a period of 1 hour. It is, of course, not claimed that the constitution of this gum is

represented above, but the evidence obtained from a study of the course of hydrolysis strongly suggests this general type of arrangement. A study of gum arabic [Norman, 1929] on similar lines led to the conclusion that it also contains a nucleus acid, resistant to mild hydrolysis, to which are attached arabinose units by a type of linkage easily broken. A similar type of arrangement has just been described for mesquite gum by Anderson and Otis [1930]. In this substance they find a resistant nucleus containing three units of galactose and one of methoxyglycuronic acid, to which are attached through a dicarbonyl linkage, four units of arabinose. It is probable therefore that this general arrangement may be found to hold for other members of this group of substances. Bassorin, which is very much more complex in structure than tragacanthin seems to be similarly constituted.

SUMMARY.

1. Tragacanthin, the soluble constituent in gum tragacanth, may be separated by ordinary filtration in extreme dilution.
2. Uronic acid units are found to be present and to constitute about one-half of the molecule. Arabinose was the only sugar found; no galactose could be detected.
3. Hydrolysis products were prepared, the analytical figures for which give rise to the suggestion that a portion of the arabinose is united to the uronic acid to form a resistant nucleus, and the residue attached by glucosidic linkage, and therefore easily removable.

REFERENCES.

- Anderson and Otis (1930). *J. Amer. Chem. Soc.* **52**, 4461.
von Fellenberg (1914). *Chem. Zentr.* ii, 943.
Hilger and Dreyfus (1900). *Ber. deutsch. chem. Ges.* **33**, 1178.
Norman (1929). *Biochem. J.* **23**, 524.
Ogle (1900). *Pharm. J.* (3), **20**, 3 T.
O'Sullivan (1901). *J. Chem. Soc.* **79**, 1164.
Tollens (1901). *Ber. deutsch. chem. Ges.* **34**, 1434.
Widstoe and Tollens (1900). *Ber. deutsch. chem. Ges.* **33**, 132.

XXVII. HYDROGENASE: A BACTERIAL ENZYME ACTIVATING MOLECULAR HYDROGEN.

I. THE PROPERTIES OF THE ENZYME.

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BIOCHEMISTS are now accustomed to regard the transfer of hydrogen as an essential step in biological oxidations; such a view involves the conception of some enzymic mechanism for rendering active or unstable the molecule from which the hydrogen is transferred. No such enzyme acting on molecular hydrogen has so far been described, though several inorganic catalysts are known which function in this way. It is nevertheless almost certain that such an enzyme exists, as organisms producing molecular hydrogen presumably have such a catalyst; moreover bacteria have long been known which are capable of oxidising hydrogen gas by molecular oxygen and living autotrophically on the energy thus liberated. The earliest example of such an organism was *B. pantotrophus* (*Hydrogenomonas pantotrophica*) isolated by Kuserer [1906]. Later other workers isolated a variety of species having the same characteristic [Niklewski, 1914; Grohmann, 1924]. It was subsequently discovered that in certain of these species nitrate can replace oxygen, so that an anaerobic autotrophic development occurs [Niklewski, 1914]. In all these cases the organisms are facultatively autotrophic, the oxidation of hydrogen playing an essential part in their metabolism.

A suggestion that these early observations come into line with recent work on the dehydrogenases of bacteria is contained in the recent work of Tausz and Donath [1930], who showed that a culture of *Bact. aliphaticum liquefaciens* grown autotrophically on hydrogen, oxygen and carbon dioxide is capable of reducing methylene blue when a stream of hydrogen is led through the apparatus, but not when nitrogen replaces the hydrogen.

Experimental.

During the study of anaerobic fermentations of fatty acids to methane by mixed cultures from river mud, a culture was obtained which (a) reduced sulphate to sulphide, and (b) decomposed formate quantitatively to methane, carbon dioxide and water. The same culture was also found to synthesise

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methane from a mixture of carbon dioxide and hydrogen and simultaneously to reduce sulphate to sulphide at the expense of hydrogen [*cf.* Söhngen, 1910]. This led to the conception that carbon dioxide and sulphate were acting as hydrogen acceptors in a system where molecular hydrogen was the hydrogen donator, and it seemed likely that bacteria were present in the mixed culture capable of activating molecular hydrogen. We therefore centrifuged a mixed culture which had been grown for some 20 subcultures on formate as sole source of carbon, and tested the washed suspension in Thunberg tubes with methylene blue and gaseous hydrogen. For this purpose hydrogen from a Kipp's apparatus was passed through three wash-bottles containing silver nitrate (for the removal of traces of volatile arsenic compounds), pyrogallol and water; the last bottle was connected with a 3-way tap (oblique bore) so that the Thunberg tube could be alternately evacuated and filled with hydrogen. The tube was evacuated thoroughly to remove dissolved oxygen, then filled with hydrogen and again evacuated and filled; finally the tap was closed and the tube was placed in a bath at 45° and shaken for 1 minute to ensure saturation of the liquid with the gas; the reduction time was measured.

Result:

	Reduction time (mins.)
1 cc. 1/25,000 methylene blue, 1 cc. phosphate buffer p_H 8.0, 1 cc. bacterial suspension, in hydrogen	15
1 cc. 1/25,000 methylene blue, 1 cc. phosphate buffer p_H 8.0, 1 cc. bacterial suspension, <i>in vacuo</i>	> 70
Control with boiled suspension in hydrogen	> 70

Having shown that our mixed culture contained organisms capable of reducing by means of molecular hydrogen, we diluted and plated on ordinary broth agar and incubated the plates anaerobically. We picked off a number of colonies, grew the corresponding cultures on broth, centrifuged and washed the organisms, and tested the suspensions as above with hydrogen and methylene blue. In these and all subsequent experiments 1 cc. of 1/5000 methylene blue in a total volume of 4 cc. was used, and the time was taken when reduction had proceeded to 75 %, because the rate of reduction appears to fall off after that point.

Result:

Culture No.	Reduction time (mins.)	
	With hydrogen	Without hydrogen
70	5½	> 120
74	> 120	> 120
79	> 120	> 120
80	5½	> 120

This shows that there is a clearly marked difference between organisms having the property of reducing by means of molecular hydrogen and those lacking the power. Culture 70 was re-plated and gave the strain known as 111.

Characteristics of organism 111. This organism is a short, motile, non-sporing, Gram-negative bacillus, growing both aerobically and anaerobically.

Sown into peptone medium with 1 % of various sugars, according to the usual bacteriological practice, the following results were obtained:

Sugar	Acid	Gas
Glucose	+	+
Fructose	+	+
Mannitol	-	+
Sucrose	-	-
Maltose	-	+
Lactose	+	+
Salicin	-	-

Other characteristics are (1) acid clot in litmus milk, (2) no liquefaction of gelatin, (3) no production of indole, (4) negative Voges and Proskauer test. We are therefore assigning this organism to the *coli-typhosus* group; its properties as far as they have been investigated agree with those of *B. formicus* (*Escherichia formica*), but its identity cannot be considered proved.

Hydrogenase. It is clear from the experiments cited already that we are dealing with a bacterial enzyme comparable with the dehydrogenases, the substrate in this case being molecular hydrogen. We must assume that the hydrogen is in some way activated, and this activation can be conveniently expressed $H_2 \rightleftharpoons 2H$ without implying anything about the nature of the reaction. In order to conform with the accepted terminology we suggest that this enzyme be called "hydrogenase."

Distribution of hydrogenase. The presence of this enzyme in what appears to be a common bacterial species made us suspect that it might be found in other well-known organisms. Various strains were grown on broth medium, centrifuged and washed, and tested in vacuum tubes in the usual way, and the following results were obtained. (To get a rough comparison between the activities of the different species, the suspensions were diluted to the same opacity.)

Strain	Reduction time (mins.)		Hydrogenase
	With H_2	Without H_2	
<i>Bact. coli</i> (Escherich)	8½	> 120	+
<i>Bact. coli</i> (Houston 1)	11½	75	+
<i>Bact. coli</i> (Houston 2)	8	> 120	+
<i>Bact. coli</i> (Houston 3)	8½	> 120	+
<i>Bact. acidilactici</i>	9½	> 150	+
<i>Pseudomon. pyocyanea</i>	65	63	-
<i>Chromobact. prodigiosum</i>	> 60	> 60	-
<i>Bac. megatherium</i>	> 120	> 120	-
<i>Bact. lactis aerogenes</i>	9½	15½	?
<i>Clostr. sporogenes</i>	> 150	> 150	-
<i>Bac. subtilis</i>	> 120	> 120	-
<i>Bact. alcaligenes</i>	27½	28	-

The relation of these results to other known facts will be discussed later.

The unexpected discovery of hydrogenase in a number of widely differing bacterial species led us to look for it elsewhere; baker's yeast and sheep's heart muscle, both normally active with regard to other dehydrogenases, were completely lacking in hydrogenase.

Properties of hydrogenase.

(a) *Effect of pressure of hydrogen.* From electrode potential measurements it has been shown that methylene blue 50 % reduced would be in equilibrium with 10^{-15} atmospheres of hydrogen at p_H 7.0 and 30° ; so that at pressures far too small for measurement we may expect methylene blue to be completely reduced provided the molecular hydrogen is activated, as it is by platinum black or by hydrogenase. This complete reduction at very low pressures naturally cannot be verified experimentally. Apart from the effect of pressure in deciding whether the methylene blue will be reduced or not, we should expect variation of pressure to affect the velocity of reduction, the upper limit of pressure at which this effect becomes noticeable being decided by the affinity of hydrogenase for hydrogen. Actually we have been unable to measure this affinity owing to other complications. In the first place, even at pressures still capable of manometric measurement, the amount of hydrogen in solution in the liquid is insufficient stoichiometrically to reduce the methylene blue present in the tube, so that the rate of diffusion of hydrogen into the liquid becomes the limiting factor; in the second place, at still lower pressures, the total amount of hydrogen in the vacuum tube is insufficient to effect the complete reduction of the methylene blue. By lowering the methylene blue concentration these difficulties could be to some extent removed, but a limit is imposed by the experimental difficulty of measuring the reduction time of very small quantities of methylene blue.

The rates of reduction of the dye were measured at different pressures of hydrogen, using the same apparatus as that described previously, except that a manometer was inserted between the 3-way tap and the pump, and a tap between the manometer and the pump. But this means the vacuum tubes could be filled with hydrogen at any desired pressure.

Two curves were obtained, one using a concentration of 1/20,000 methylene

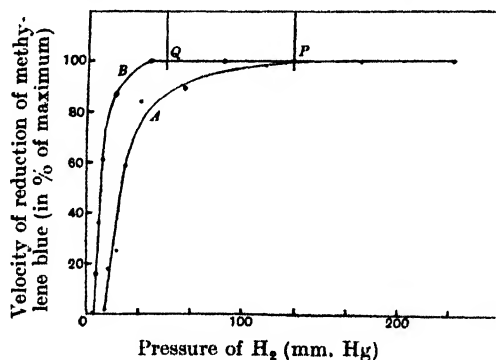


Fig. 1.

blue (Fig. 1, curve A), the other using 1/50,000 (curve B). From the solubility of hydrogen at 45° and the molecular weight of methylene blue it is calculated

that at a pressure of 130 mm. (*P*) just sufficient hydrogen is dissolved to reduce the higher concentration of methylene blue, and that at 50 mm. (*Q*) there is enough dissolved for the reduction of the lower concentration (*cf.* curves *A* and *B*). Hence the pressure-velocity curves give no measure of the affinity of the enzyme for its substrate, but serve to show that by working at a pressure of one atmosphere we are certain that the enzyme is saturated.

(*b*) *Effect of hydrogen ion concentration.* The relation between hydrogen ion concentration and velocity of reduction of methylene blue has been determined in the case of two organisms, viz. *Bact. coli* (Escherich) and strain 111. The results are given in Figs. 2 and 3, each compiled from the results of two separate experiments.

Alcock and Cook [personal communication] have determined the p_H curves of several bacterial dehydrogenases, and they find that after the characteristic increase of velocity up to a maximum at p_H 6.0 the velocity remains constant up to p_H 9.0, *i.e.* until irreversible destruction of the enzyme takes place in

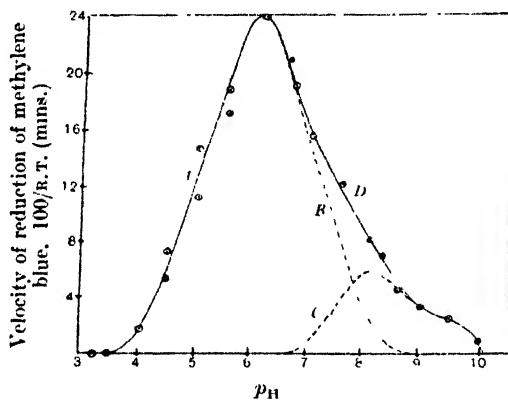


Fig. 2. *Bact. coli* (Escherich).

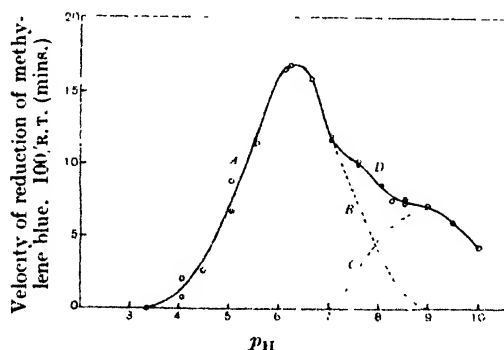


Fig. 3. Strain 111.

the alkaline region. In this respect hydrogenase differs very greatly from these dehydrogenases. Bernheim however [1928] described an aldehyde oxidase

from potato whose p_H -activity curve with respect to methylene blue showed a very clear peak with its optimum at p_H 6.8.

The shoulder on the descending part of the curve is difficult to explain; it is possible to analyse it into two dissociation residue curves, as shown by the dotted lines in the diagram. The ascending part of the curve *A* is identical in the case of the two organisms; the descending line *B* is obtained by drawing a curve symmetrical with *A*; the points on the line *C* are obtained by subtracting the values on the hypothetical curve *B* from the corresponding points on the experimental curve *D*. Whether this may be regarded as evidence for the existence of two hydrogenases having different p_H optima is a matter we are not prepared to discuss; it is noteworthy, however, that the optimum on the first peak is at about p_H 6.3 and that on the second at about p_H 8.5 in both organisms.

(c) *Effect of cyanide*. There is obviously an analogy between the activation of hydrogen by hydrogenase and that of the hydrogen atoms of carbon compounds by dehydrogenases. This similarity is borne out by the action of potassium cyanide, as the following experiment shows.

In order to show the effect of cyanide on the rate of reduction of methylene blue in a vacuum tube it is necessary to guard against the loss of hydrogen cyanide during the evacuation. For this purpose vacuum tubes with hollow stoppers were employed, the potassium cyanide solution being placed in the stopper at p_H 12; the contents of the tubes themselves were strongly buffered at p_H 6.0, and it was found by trial that when the contents of the stopper were mixed with those of the tube the p_H of the resulting mixture was not above 6.5, *i.e.* was still within the optimum range.

Results:

Concentration of KCN	Reduction time (mins.)
0	7
<i>M</i> /800	5
<i>M</i> /240	4½
<i>M</i> / 80	4
<i>M</i> / 24	3

(d) *Effect of narcotics*. Keilin [1929] has shown that whereas the inhibition of respiration produced by cyanide is due to inhibition of the indophenol oxidase, that produced by urethane is to be attributed to inhibition of the reducing systems of the cell, *viz.* the dehydrogenases. The following figures show that hydrogenase is also inhibited by urethane:

Conc. of urethane (%)	Reduction time (mins.)	% inhibition
0	4½	0
0.025	7½	41
0.25	8½	51.5
2.5	11½	64

In its reactions to cyanide and urethane hydrogenase behaves as a typical dehydrogenase.

Reduction of other hydrogen acceptors.

One would expect that any substance that can be reduced by a given organism with the aid of reducing substances will also be reduced by it with molecular hydrogen provided that the organism possesses hydrogenase. This has been tested by means of the Barcroft differential manometer in the cases of *Bact. coli* and strain 111.

Reduction of molecular oxygen. For this purpose the right-hand cup of the apparatus was separately evacuated and filled with a gas mixture containing 20 % oxygen and 80 % hydrogen. The left-hand cup remained filled with air. Each cup contained 3 cc. of bacterial suspension in phosphate buffer. A typical result is shown in Fig. 4, curve 1. It is seen that in this case the activity of the enzyme falls off rapidly; we first suspected the formation of hydrogen peroxide but failed to demonstrate it by the peroxidase and guaiacum test. It seems however that mere shaking with air inactivates the enzyme; this we demonstrated by removing the contents of the left-hand Barcroft cup and placing it in a vacuum tube with methylene blue and adding hydrogen in the usual way. The reduction time was > 1 hour, while that of the untreated control was 7 mins.

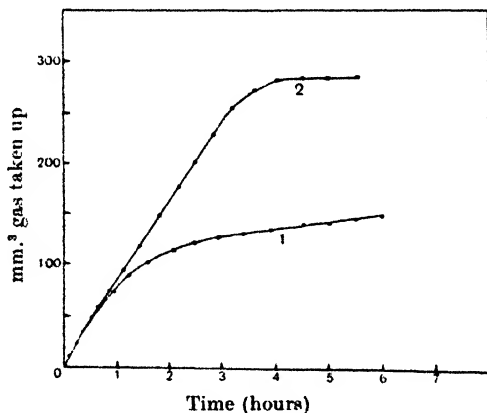


Fig. 4. 1. $H_2 + O_2$. 2. Nitrate in H_2 .

Reduction of nitrate. For these experiments both cups of the Barcroft apparatus were completely evacuated and filled with hydrogen. Potassium nitrate ($M/1000$ – $M/300$) was present only in cup 2. The uptake of hydrogen proceeded linearly, but never attained completion; Fig. 4, curve 2, gives a typical result, the hydrogen here taken up corresponding to 53 % reduction of the nitrate to nitrite. Estimation of the nitrite at the end of the experiment, however, showed that the nitrate was completely reduced. A further addition of nitrate (in a companion experiment) showed that reduction again proceeded to the same extent, though slightly more slowly, and the same was true of a third and fourth addition (Fig. 5). The explanation of this phenomenon was obtained by carrying out parallel experiments in vacuum tubes where no

hydrogen was present. Here it was found that the nitrate was also reduced by the donors of the cell alone; hence in the Barcroft cup we have the result of a competition for the nitrate between the donors of the cell and the hydrogen.

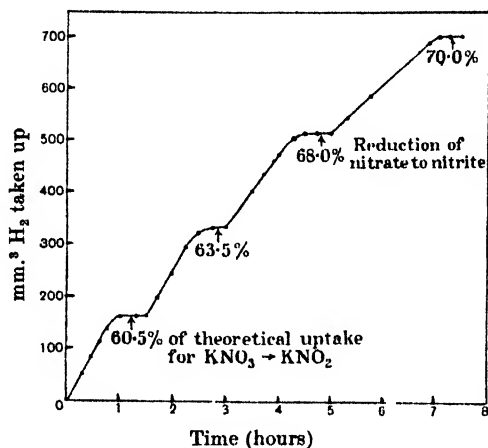


Fig. 5.

Exp.		Nitrate used (<i>M</i>)	Nitrite found (<i>M</i>)	H ₂ uptake (% of theory for N(O ₃)' → N(O ₂)')
1	In hydrogen	0.0040	0.0044	41.0
2	"	"	0.0043	33.0
3	"	0.0080	0.0070	39.5
4	"	"	0.0072	42.5
5	<i>In vacuo</i>	0.0040	0.0042	—
6	"	"	0.0037	—
7	"	"	0.0040	—

We attempted to obtain a reduction of nitrate by hydrogen and hydrogenase dissociated from reductions by cell substances; for this purpose we incubated the cell suspension with a 0.3 % solution of potassium nitrate for varying periods; the resulting nitrite was then removed by centrifuging and the suspension was tested as before with fresh nitrate in the Barcroft apparatus. This was however unsuccessful as the preliminary treatment so damaged the hydrogenase that the rate was greatly reduced and the hydrogen uptake was even less complete than when the untreated suspension was used.

Reduction of fumarate. The reduction of fumarate by hydrogen was shown in the same way as that of nitrate, *M*/30 to *M*/120 fumarate being used in place of nitrate. Hydrogen was taken up linearly but the uptake ceased when only about 30 to 60 % of that required for the complete reduction to succinate had disappeared. This incomplete uptake is probably due to the same cause as in the case of nitrate, *i.e.* to a part of it being reduced by the hydrogen donors of the cell; possibly also the anaerobic decomposition of fumarate occurring alongside the reduction may be a contributory cause.

The reduction of bacterial substance. It is well known that bacteria, in common with other living tissues, effect two kinds of oxidation, an endogenous

oxygen uptake in which the cell substance alone is concerned [Callow, 1924] and a much more rapid exogenous uptake when an oxidisable substance is added [Cook and Stephenson, 1928]. We therefore sought to discover whether, in addition to the exogenous reductions already demonstrated as the result of the action of hydrogenase, an endogenous reduction of cell material could also occur. For this purpose 3 cc. of a suspension of *Bact. coli* in buffer at p_H 6.5 was placed in the right-hand Barcroft cup and 3 cc. of buffer alone in the left-hand cup; both cups were evacuated and filled with hydrogen; no hydrogen uptake was observed. We therefore conclude that the cell has no store of hydrogen acceptors, and therefore no endogenous reduction occurs comparable with cell respiration.

*The relation of hydrogenase to dehydrogenases and its function
in cell metabolism.*

From its general character it seems justifiable to regard hydrogenase as an enzyme closely related to the dehydrogenase class. It also seems likely that the enzyme which catalyses the change of molecular to activated hydrogen catalyses also the reverse reaction, viz. the production of molecular hydrogen from activated hydrogen atoms, that is, the reversible reaction, $H_2 \rightleftharpoons 2H$, should be catalysed in both directions. Previous studies by one of us [Stickland, 1929] have made it clear that the production of molecular hydrogen from formate is the work of two enzymes; it was there shown (1) that non-gas-producing organisms (e.g. *Bact. typhosum*) may possess an active formic dehydrogenase, (2) that in the case of *Bact. coli*, which possesses the double mechanism, treatment with trypsin augments the dehydrogenase and destroys the mechanism producing gaseous hydrogen. We therefore tentatively suggest that the production of molecular hydrogen from formate is the work of the two enzymes formic dehydrogenase and hydrogenase. Such a hypothesis would of course be invalidated if an organism could be found able to produce hydrogen from formate yet not possessing hydrogenase. So far, among the organisms investigated, we have not come across a definite exception of this sort; the following list illustrates this. In the case of *Bact. lactis aerogenes* the presence of

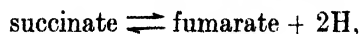
Organism	Ability to produce hydrogen from formate	Presence of hydrogenase
<i>Bact. coli</i> (Escherich)	+	+
<i>Bact. coli</i> (Houston 1)	+	+
<i>Bact. coli</i> (Houston 2)	+	+
<i>Bact. coli</i> (Houston 3)	+	+
<i>Bact. acidi lactici</i>	+*	+
<i>Bact. lactis aerogenes</i>	+	?
<i>Bact. alcaligenes</i>	—	—
<i>Ps. pyocyanea</i>	—*	—
<i>Chromobact. prodigiosum</i>	—†	—
<i>Bac. megatherium</i>	—*	—
<i>Bac. subtilis</i>	—*	—

* Pakes and Jollyman [1901].

† Pakes and Jollyman obtained a positive result with *Chromobact. prodigiosum*, but our strain, tested by ourselves, gave completely negative results.

hydrogenase is not thoroughly established, the reduction of methylene blue being slow and sometimes absent; we propose to investigate this point further.

According to the view suggested above, we should expect organisms possessing hydrogenase to liberate molecular hydrogen from other donators; two conditions at least would however have to be satisfied before this could occur. First, no hydrogen acceptors must be present since these would presumably seize the hydrogen before it was liberated; second, the r_H of the system must be such that the hydrogen would be liberated at some measurable pressure, *i.e.* $r_H \succ 3$, say. For instance, in the case of



the theoretical pressure of hydrogen in equilibrium with the system is of the order of 10^{-8} atmospheres.

SUMMARY.

1. An enzyme has been found in a number of bacterial species which activates molecular hydrogen; for this the name "hydrogenase" is suggested.
2. By means of this enzyme hydrogen reduces molecular oxygen, methylene blue, nitrate and fumarate.
3. The properties of this enzyme are described, and its relation to other bacterial enzymes and also its function in the cell are discussed.

We wish to record our thanks to Dr Malcolm Dixon for very useful criticism in connection with this work, and to Sir Frederick Hopkins for his kind interest and advice.

REFERENCES.

- Bernheim (1928). *Biochem. J.* **22**, 344.
Callow (1924). *Biochem. J.* **18**, 507.
Cook and Stephenson (1928). *Biochem. J.* **22**, 1368.
Grohmann (1924). *Zent. Bakt. Par.* **11**, 61, 256.
Kaserer (1906). *Zent. Bakt. Par.* **11**, 16, 681.
Keilin (1929). *Proc. Roy. Soc. Lond. B* **104**, 206.
Niklewski (1914). *Zent. Bakt. Par.* **11**, 40, 430.
Pakes and Jollyman (1901). *J. Chem. Soc.* **79**, 386.
Söhngen (1910). *Rec. Trav. Chim.* **29**, 238.
Stickland (1929). *Biochem. J.* **23**, 1187.
Tausz and Donath (1930). *Z. physiol. Chem.* **190**, 141.

XXVIII. HYDROGENASE.

II. THE REDUCTION OF SULPHATE TO SULPHIDE BY MOLECULAR HYDROGEN.

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(Received December 31st, 1930.)

IN a previous communication [Stephenson and Stickland, 1931] it was shown that many common species of bacteria possess an enzyme which activates molecular hydrogen; this enables them to reduce various hydrogen acceptors by means of hydrogen gas. It was there mentioned that the mixed culture, obtained from the River Ouse, in which this was first observed was able to reduce sulphate to sulphide by molecular hydrogen, and this reaction forms the subject of the present paper.

It was originally shown by Beijerinck [1895] that the hydrogen sulphide produced in mud arises anaerobically by the bacterial reduction of sulphates. Van Delden [1904] first obtained pure cultures of the organisms concerned (*V. desulphuricans* and *V. aestuarii*) in a medium in which sulphate was reduced to sulphide anaerobically at the expense of organic compounds (lactate and malate), the energy for development being derived from the reduction of the sulphate. Elion [1924] isolated a thermophilic organism (*V. thermodesulphuricans*) of the same type. Quite recently an exhaustive study of the metabolism of the sulphate-reducing bacteria has been made by Baars [1930]. Using several strains he investigated the products of oxidation of a large number of carbon compounds, and showed that the strains differed in the compounds which they were able to oxidise.

With regard to the reduction of sulphate by molecular hydrogen the only reference which we have been able to find is a statement by Niklewski [1914], who claims to have isolated an organism able to carry out this reaction, but gives no details.

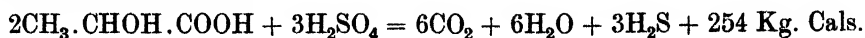
Isolation and description of the organism.

In order to isolate the organism from the mixed culture, the technique employed by Van Delden was used; this consists in plating on broth agar containing sulphate and iron salts, the concentrations used being 0.5 % sodium sulphate and 0.005 % potassium iron sulphate. The plates were incubated anaerobically, and the colonies responsible for the production of sulphide were

¹ Goldsmiths' Senior Student and Benn W. Levy Student.

distinguished by being jet black owing to the precipitation of iron sulphide. After several repetitions a culture was obtained which gave only black colonies; the pure culture was known as Strain 182.

Like the other sulphate-reducers described in the literature this organism can be grown anaerobically on an inorganic medium containing sulphate and lactate, thus proving that it derives its energy from the oxidation of the lactate by the sulphate:



It also resembles *V. desulphuricans* in being a small comma-shaped organism, strictly anaerobic and non-spore-forming.

It has been shown by Baars that the different species of sulphate-reducers, while agreeing in their general morphological and physiological characteristics, differ among themselves in their abilities to use various carbon compounds for the reduction of sulphate. We therefore tried to establish the identity of our Strain 182 by sowing it into a number of media containing a variety of carbon compounds and sulphate. The medium [Baars, 1930] consisted of

				%
K_2HPO_4	0.05
NH_4Cl	0.1
CaSO_4	0.1
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2
FeSO_4	Trace

with 30 millimols of carbon compound per litre, and the results were as follows:

Carbon compound	<i>V. desulph.</i>	<i>V. rübensch.</i>	Strain 182
Glycerol	+	+	-
Mannitol		+	-
Glucose	+	+	-
Fructose	+	+	+
Galactose	-	+	?
Sucrose	-	+	-
Lactose	-	+	-
Maltose	-	+	-
Formic acid	+	+	+
Acetic „	-	+	-
Butyric „	-	+	-
Lactic „	+	+	+
Tartaric „	-	+	-
Succinic „	+	+	-

We thus have to conclude that Strain 182 is not identical with any organism studied by Baars.

Presence of hydrogenase in Strain 182.

The bacteria were grown on 1200 cc. of a medium containing 25 % of tryptic broth, 0.5 % sodium formate, 0.5 % sodium sulphate and 50 % of inorganic medium [Stephenson, 1930]. After about 5 days' anaerobic incubation, the cells were separated from the medium by centrifuging, washed twice

with Ringer's solution and suspended in 50 cc. of Ringer's solution. By the method already described [Stephenson and Stickland, 1931] it was proved that this strain actively reduces methylene blue by molecular hydrogen, *i.e.* contains hydrogenase.

	Reduction time (mins.)
Suspension (diluted 1 in 5) <i>in vacuo</i>	> 180
" " in hydrogen	4
Boiled suspension in hydrogen	> 180

The ability to use hydrogen is not a peculiarity of our strain, as it was found by the same method that *V. rübentschickii* also contains hydrogenase.

	Mins.
Reduction time <i>in vacuo</i>	> 150
" " in hydrogen	17

Reduction of sulphate by molecular hydrogen.

(a) *Qualitative.* 1 cc. of a washed suspension of bacteria of Strain 182 was incubated at 37° for 48 hours with 1 cc. of 0.6 % sodium sulphate in phosphate buffer at p_H 7.4, in hydrogen and *in vacuo*. In the tubes containing hydrogen a strong smell of hydrogen sulphide and a strong reaction with nitroprusside were observed, and in the controls both tests were negative. By the same method the reduction of sulphate by hydrogen in presence of *V. rübentschickii* was demonstrated.

(b) *Quantitative.* The apparatus (Fig. 1) consisted of a bolt-headed flask of

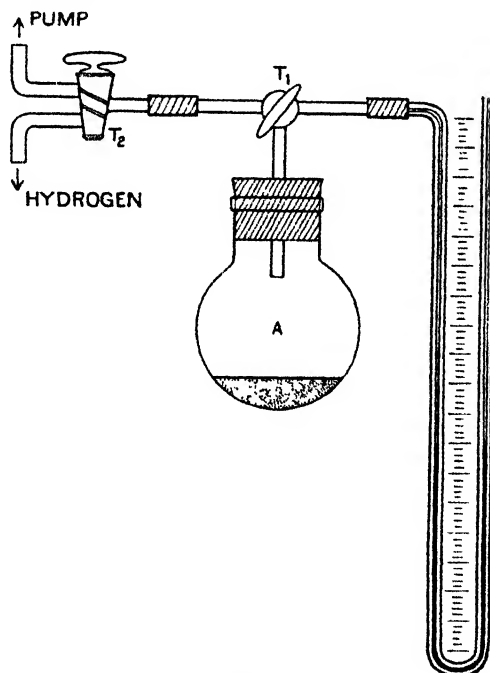


Fig. 1.

200 cc. capacity (*A*), fitted with a 3-way tap T_1 ; the latter was connected with a manometer. Into *A* were put 10 cc. phosphate buffer, p_H 7.2, 10 cc. of standard solution of sodium sulphate and 20 cc. of suspension of the organism. Before placing the stopper in the flask the tap T_1 was opened to the manometer, which was tipped till the mercury reached the tap; the latter was then closed by a turn through 90° . The rubber bung was then inserted in the flask, and its lower level marked on the glass. The distal tube of T_1 was then connected to a 3-way tap T_2 , by which it could be joined either to a water-pump or to a supply of hydrogen. The flask was first evacuated, the liquid being shaken to expel dissolved air, and then filled with hydrogen, this process being repeated twice to ensure thorough removal of oxygen. T_2 was then closed, T_1 turned so as to connect the flask with the manometer and the flask disconnected. The whole was then placed in the incubator at 34° . When the temperature had become constant as shown by the manometer, the manometer and barometer readings were simultaneously taken, and these readings were taken at suitable intervals during the experiment until the absorption of hydrogen was complete (see Table I).

Table I.

Date (August)	Time	Manometer diff.	Uptake	Correct. for change in bar.	Uptake (corrected) mm. Hg
19	9.0 p.m.	+ 50.0	0.0	0.0	0.0
20	9.30 a.m.	+ 1.0	49.0	- 1.5	47.5
20	11.30 p.m.	- 13.0	63.0	- 1.5	61.5
21	10.0 a.m.	- 20.5	70.5	+ 14.5	85.0
21	10.30 p.m.	- 40.0	90.0	+ 8.5	98.5
22	10.0 a.m.	- 58.0	108.0	+ 1.5	109.5
22	10.30 p.m.	- 63.5	113.5	- 0.5	113.0
23	9.30 a.m.	- 66.0	116.0	+ 6.5	122.5
24	10.0 "	- 73.0	123.0	- 0.5	122.5
25	10.0 "	- 79.0	129.0	- 6.5	122.5

The stopper was then removed and the contents of the flask made alkaline by the addition of 10 cc. of *N* NaOH. Two 10 cc. samples were placed in 300 cc. flasks and acidified, and the hydrogen sulphide blown from them by a current of nitrogen into a series of three small absorption bottles, the first two each containing 10 cc. of *N*/50 iodine, the third about 10 cc. of toluene to absorb the iodine carried through by the current of gas. After 4 hours the iodine was titrated with *N*/50 thiosulphate, and the amount of hydrogen sulphide calculated. The residual sulphate, after removal of bacterial debris by filtration through kieselguhr, was estimated for us by a micro-method by Mr A. Colwell. The volume of the gas space in the flask and the tubes was measured.

Calculation of results.

(a) *Hydrogen used.* Change of pressure (corrected for change of barometer) = 445.5 mm. Hg.

Volume of gas space in flask = 134.0 cc., temperature, 34° .

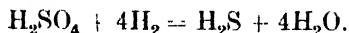
Hydrogen used = $\frac{134 \times 445.5 \times 273}{760 \times 307} = 69.9 \text{ cc.} = 3.12 \text{ millimols.}$

(b) *Hydrogen sulphide produced.* Iodine used by total volume = 73.7 cc. $N/50 = 36.85$ cc. $M/50$ $H_2S = 0.74$ millimols.

(c) *Sodium sulphate reduced.* The Na_2SO_4 added, 0.100 g. = 0.70 millimols, was completely reduced.

Results.

As four molecules of hydrogen are required to reduce one molecule of sulphate to sulphide, the figures for hydrogen in the table are divided by four in order to show the extent of the agreement:



Exp.	Sulphate reduced (millimols)	Hydrogen used	Sulphide formed (millimols)
		4 (millimols)	
1	0.70	0.78	0.74
2	0.40	0.38	0.35

Control experiments. (a) An exactly similar experiment to the above, without the addition of sulphate, showed no uptake of hydrogen. (b) A suspension of cells incubated with sulphate in nitrogen instead of hydrogen gave no hydrogen sulphide. (c) An identical experiment carried out with full sterile precautions, both in the centrifuging and washing of the organisms and in the setting up of the apparatus, gave similar results, and showed no contaminating colonies on plating at the end of the experiment.

Reduction of other hydrogen acceptors.

Baars showed that the various strains of *V. desulphuricans*, when sown into lactate synthetic medium containing sulphate, sulphite, hyposulphite, thiosulphate or sulphur, reduced them all to sulphide. We found that the washed suspension of cells of Strain 182 reduced sulphate, sulphite and thio-sulphate to sulphide in the presence of hydrogen and not in the control experiments with hydrogen absent.

Effect of hydrogen ion concentration.

As hydrogenase was first studied in a group of organisms differing widely in their physiology from Strain 182, it seemed worth while to ascertain whether the enzyme showed any marked divergence of properties in association with this sulphate-reducing species. For this reason we compared the effect of hydrogen ion concentration on the enzyme of this organism with that on the enzyme in *Bact. coli* and Strain 111 previously recorded [Stephenson and Stickland, 1931]. The result is shown in Fig. 2; the explanation of the dotted lines and the lettering will be found in the earlier paper [1931, p. 210]. The curve is the same in its main character as those obtained with the other species, and it seems probable that we are dealing with the same enzyme.

The peak at p_H 6.3 gives the optimum reaction for the activation of

hydrogen; we have at present no accurate method for determining the optimum p_H for the activation of sulphate, so that the optimum for the complete reaction is unknown.

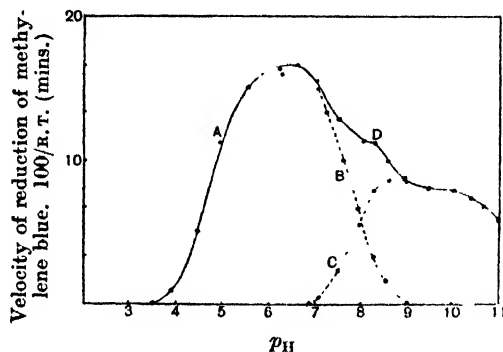


Fig. 2.

SUMMARY.

1. The enzyme hydrogenase has been found in a typical sulphate-reducing organism from the River Ouse.
2. This organism is able to reduce sulphate quantitatively to sulphide by means of molecular hydrogen according to the equation

$$\text{H}_2\text{SO}_4 + 4\text{H}_2 = \text{H}_2\text{S} + 4\text{H}_2\text{O}.$$
3. Sulphite and thiosulphate are also reduced to sulphide.

We wish to record here our thanks to Prof. A. J. Kluyver of Delft for supplying us with strains of *V. desulphuricans* and *V. rubentschickii*.

One of us (L. H. S.) is indebted to the Department of Scientific and Industrial Research for a grant.

REFERENCES.

- Baars (1930). Over het sulphaat reductie door bacterie. (Dissertation, Delft.)
 Beijerinck (1895). *Zent. Bakt. Par.* II, 1, 1.
 Van Delden (1904). *Zent. Bakt. Par.* II, 11, 81, 113.
 Elion (1924). *Zent. Bakt. Par.* II, 63, 58.
 Niklewski (1914). *Zent. Bakt. Par.* II, 40, 430.
 Stephenson (1930). *Bacterial metabolism.* (Longmans, London.)
 Stephenson and Stickland (1931). *Biochem. J.* 25, 205.

XXIX. THE EFFECT OF INSULIN ON GROWTH, NITROGEN EXCRETION AND RESPIRATORY METABOLISM¹.

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(Received December 24th, 1930.)

THE clinical observation that insulin therapy in diabetes is frequently associated with a rapid increase in weight has led to considerable discussion as to whether the hormone promotes the formation of fat from carbohydrate or allows of a greater degree of sparing of fat and protein by a more complete metabolism of carbohydrate. In the diabetic subject the increase in weight is frequently brought about even when the diet is maintained at a level not necessarily in excess of what would be regarded as normal for age and weight. With the normal animal the matter is not clear. Whilst Macleod *et al.* [1930] refer to rabbits which gain weight rapidly during utilisation for the assay of insulin, Long and Bischoff [1930] could find no effect of insulin on the growth curves of these animals. The former investigators using young pigs and doses of insulin from 1.5 to 2 units per kg. could observe no very striking effect on the rate of increase in weight.

In view of these findings it is perhaps of interest to report our findings with young albino rats. In the following experiments very much larger doses of insulin were used than would *a priori* be considered compatible with the survival of the animal.

The animals were bred in the laboratory from a single pair and were of an extremely healthy and uniform strain. Comparisons were made between litter-mates only.

The method of experiment was to subject the animals of one series to a restricted diet, making certain by hand feeding that each individual received the same quantity daily. Another series was investigated with an unlimited quantity of food. Thus the appetite factor was of significance only in the second series. The diet consisted in every case of a mixture of white bread and fresh whole milk. No attempt was made to keep the diet of absolutely constant composition, but the average composition was 0.74 g. N and 17.74 g. carbohydrate (as glucose after hydrolysis) per 100 g. mixture and each animal of the restricted series received the same amount on any given day.

¹ Work performed during tenure of a Beit Memorial Research Fellowship by M. W. G.

In the case of the animals kept on an unrestricted amount of food, care was taken that the container was always well filled. The animals were all isolated in separate compartments. The doses of insulin, administered subcutaneously, varied in the animals on a restricted diet from 0.25 units to 40 units daily, always injected in one dose. In the case of the series on an unrestricted diet, the doses varied from 2 to 50 units daily, also given at one time.

The insulin used was that prepared by Messrs Burroughs Wellcome and Co.

Considerations of space allow only averages to be given, but it is to be understood that the growth curves of individual animals were practically identical.

Table I.

Litter 1. Restricted diet. Born 16. ii. 30. Weights given in g.													% increase in wt.
Age in days	26	27	30	32	34	37	40	43	46	50	55	61	—
Controls (wt. in g.) (1 ♀, 1 ♂)	49	53.5	55.5	58	59.5	65.5	70	74	81.5	87	100	111	126
Insulinised (1 ♂, 1 ♀)	45.7	50	50.3	50.3	53	63	69	73	83	89	107	109	138
Litter 2. Restricted diet. Born 18. ii. 30.													
Age in days	24	25	28	30	32	35	38	41	44	48	53	59	-
Controls (wt. in g.) (1 ♂, 3 ♀)	47	48	48.5	50	50	63	71	77	83.5	89	112	113	139
Insulinised (♀, ♂)	47	48	51	50	61	69	69	73	84	88	101	106	136
Insulin dosage for rats on restricted diet.													
Days	0-4	5-9	10-12	13	14-18	19-20	21-24	25-26	27	28	29-35		
Units per day	0.25	0.5	1.0	2	8	12	16	20	30	40	30		
Litter 3. Unrestricted diet. Born 28. vi. 30.													
Age in days	20	24	25	30	36	42							—
Controls (wt. in g.) (3 ♂, 3 ♀)	42	64	80	98	111	107							198
Insulinised (2 ♂, 4 ♀)	37	56	72	90	105	115							212
Litter 4. Unrestricted diet. Born 28. vi. 30.													
Controls (wt. in g.) (2 ♂, 1 ♀)	38	62	82	101	114	131							249
Insulinised (1 ♂, 2 ♀)	36	60	74	95	106	123							238
Insulin dosage for rats on unrestricted diet.													
Days	0-4	5-7	8	9	10-11	12-13	14-19	19-22					
Units per day	0	2	4	6	10	20	40	50					

Note. Of the insulinised animals, two in litter 1 and one in litter 2 were killed on the 61st and 55th days respectively by allowing them to consume all their diet in a short time and then injecting the insulin.

It will be observed from the results in Table I that the rate at which the animals put on weight was very much greater in the case of those on an unrestricted diet. Whereas the controls on the restricted diet showed an increase of about 130 % in 35 days, those on the unrestricted diet increased by over 200 % in 22 days. In this way a large range of growth activity was

obtained and any effects which insulin might have been able to produce could have been interpreted as having a broad application.

That no tolerance to large doses of insulin had developed was shown by the fatal effect of a dose of 50 units of insulin after withdrawal of food (see *Note*, Table I).

In administering such large doses of insulin, one has to consider the question of the degree to which it is eliminated in the urine. Whilst this may have accounted for a certain amount of loss, there can be little doubt that the animals were liable to a fatal hypoglycaemia during the greater part of the day, and were able to prevent its occurrence only by frequent feeding.

It is therefore not a little surprising that the hormone had, in fact, no effect whatever on either the rate of growth or on the final percentage increase in weight. Whatever theory may be held as to the essential action of insulin in the normal animal, these results appear difficult to reconcile with it. If the essential effect be regarded as a stimulus to peripheral oxidation of carbohydrate with a simultaneous drain on the glycogen of the liver, then it would have been reasonable to expect that at least in the case of the animals on the restricted diet, either a loss of weight or a diminished rate of increase in weight would have occurred. If, on the other hand, we regard the action of insulin as an inhibition of glycogenolysis, then we should have to suppose a temporary inhibition of the release of glycogen from the liver but a complete and adequate readjustment when the excess of the hormone had been eliminated. The experiments of Chaikoff and Macleod [1927] showed that rabbits on a high carbohydrate diet exhibited no increase in metabolism under the action of insulin. Lublin [1926], however, stated that, in man, simultaneous administration of carbohydrate and insulin might cause the R.Q. to exceed unity, and this he interpreted as evidence of a transformation of carbohydrate to fat. In the many experiments one of us (M. W. G.) has carried out on human subjects he has never seen such a phenomenon. Insulin indeed cannot increase the rate of metabolism and R.Q. above certain levels. When such increase cannot occur, a fall in blood sugar would only be possible if the release of carbohydrate from the liver were inhibited. Such a view is however not borne out by the fact that administration of insulin to a rat causes no increase in its hepatic glycogen, and may indeed, during active absorption, actually produce a fall.

In the present state of our knowledge it would be premature to attempt to reconcile these matters with our total inability to demonstrate a modified rate of growth by the action of insulin. Experiments on the effect of insulin on the protein metabolism of rats during starvation have demonstrated no increased protein catabolism. Since the largest dose of insulin which could be safely given was 0.5 unit it was however difficult to obtain a conclusive result. In the following experiments the plan was to subject rats to a period of 3 days' starvation, making daily collections of urine. The animals were then allowed to feed until the original weight was regained (always much more

than 3 days) after which a second period of starvation was begun during which insulin was administered. The fatalities were sufficiently frequent to make the experiment rather tedious. The urine was in most cases analysed for total N, inorganic P and creatinine.

Table II.

Day	Average initial wt. g.	Average daily excretion per 100 g. initial wt.			Remarks
		Nitrogen	Inorganic P	Creatinine	
		g.	mg.	mg.	
(a) Control periods of starvation. Results from 14 rats in pairs. Average:					
1	424	0.070	5.9	4.6	Starvation
2	—	0.057	6.5	4.1	„
3	—	0.064	6.5	3.9	„
(b) Periods of starvation and insulin. Results from 3 pairs from above:					
1	389	0.099	4.4	3.5	Starvation
2	—	0.107	8.3	3.1	Starvation and insulin
3	—	0.060	5.2	2.2	„

The doses of insulin were 0.5 unit per animal daily.

On the second day one of the animals died in hypoglycaemia; exp. continued with the remaining two pairs. On the third day all the animals were hypoglycaemic; it was thought necessary to give one pair a few drops of sugar solution *per os*; hence drop in N excretion. No convulsions at any time.

Table III.

Larger doses of insulin.

Three adult rats were used and subjected to a preliminary starvation of 3 days, allowed to recover their original weight and then starved and treated with insulin.

Day	Initial period of starvation			Insulin period		
	1	2	3	1	2	3
Insulin daily (units)	—	—	—	1.5	1.0	1.0
Average N per 100 g. initial wt.	0.070	0.057	0.057	0.040	0.062	0.083

On the first day one animal died in hypoglycaemia, continued with remaining two.

We think that these results (Tables II and III) justify the conclusion that, in the starving normal rat, even in hypoglycaemic doses, insulin does not increase the catabolism of protein. When, however, as in pair 3 on the third insulin day (Table II), glucose is given, there occurs a very sharp drop in excreted nitrogen. The incidence of convulsions would of course increase the general catabolism, but in the above experiments these did not occur, so that our remarks apply to the primary effect of insulin and not to the secondary manifestations of hypoglycaemia.

In spite of the very large literature on the effects of insulin on metabolism, it cannot be said that we are clear as to its essential action. The difficulty of dissociating its effects from those of other hormones acting simultaneously in the intact animal has encouraged some workers, notably Dale and his collaborators, in the use of eviscerated and decapitated preparations. The relationship of their preparation to the living animal must however be remote. Effects can be observed to follow the injection of insulin into normal animals which

seem to be irreconcilable with the results obtained in the perfused preparation. Thus, in the latter glucose disappears from the perfusate, the oxygen utilisation rises very considerably and synthesis of glycogen occurs in the muscles, the R.Q. of the preparation remaining unity throughout. In the intact animal on a high carbohydrate diet, however, administration of insulin produces no rise in R.Q. or oxygen utilisation, whilst in the starving animal, insulin injection is followed by a rise in R.Q. and a definite rise in oxygen utilisation. Further in certain species of animal a very large rise in liver glycogen can be demonstrated to follow relatively large doses of insulin during starvation, whereas in others a definite loss occurs. The matter becomes even more confusing when we add the above findings that enormous doses of insulin do not affect the rate of growth of normal rats on restricted or unrestricted diet or their excretion of nitrogen in starvation. These latter facts, it seems to us, make it clear that the rat possesses mechanisms by means of which the tendency for insulin to increase the peripheral oxidation of sugar is effectively balanced by a diminished utilisation of other substances, probably fat. In other words, the effect of insulin is to produce a diversion of metabolism in the direction of carbohydrate. This causes a rise in R.Q. and may or may not produce an increase in total metabolism according to the readiness with which these mechanisms are brought into action. Thus, as will be demonstrated in the human subject, a rapid increase in heat production and R.Q. can occur after injection of insulin; but a progressive fall in blood-sugar may also occur without any such rise in R.Q. or metabolic rate (*v. infra*). It seems to us that this can only be explained by attributing to insulin the double action of inhibiting glycogen release from the liver and stimulating peripheral oxidation, these effects not occurring simultaneously. The rat appears normally to possess extraordinarily active mechanisms for the mobilisation of its hepatic glycogen for the needs of its peripheral tissues, so that the administration of insulin is never followed by a demonstrable rise in liver-glycogen, such as has been found both in the young cat and rabbit [Goldblatt, 1930]. It is perhaps relevant here to describe certain experiments carried out on young dogs to illustrate that the latter effect is not as unusual as the recent work of Corkill [1930] might lead us to believe.

The methods used were those described by one of us (M. W. G.) in previous communications. Comparisons were made on litter mates. The insulinised animals were always killed before any symptoms occurred. The values for muscle-glycogen were obtained from a mixed sample from all four limbs.

It is to be observed that the effect here described is by no means as striking as that found in young rabbits nor are the control values as uniform as is desirable in such comparison experiments. But it is clear that, as in young cats and rabbits, a dangerous hypoglycaemia can occur even when large amounts of glycogen are still available in the liver and before any marked change has occurred in the muscle glycogen. In so far as we are justified in taking averages of these rather divergent figures, the conclusion may be drawn

The effect of insulin on young dogs.

Animal No.	Wt. g.	Blood sugar g. per 100 cc.	Plasma inorg. P mg. per 100 cc.	Glycogen			Remarks
				Liver		Muscle %	
				%	Total mg.		
Litter 1. 24 hrs. starvation.							
1	530	0.102	8.60	0.84	140	0.82	Control
2	510	0.104	7.93	1.47	265	0.69	"
3	375	0.095	7.86	2.06	258	0.44	"
			Av. 1.46		Av. 0.65		
4	576	"0"	6.46	2.21	417	0.53	11.20 a.m. 0.5 unit 12.45 p.m. 0.5 1.40 1.0 2.25 Killed
5	600	0.037	—	2.22	460	0.66	11.23 a.m. 0.5 12.45 p.m. 0.5 1.40 1.0 2.10 Killed
6	560	0.077	6.60	1.73	347	0.78	12.05 p.m. 0.5 1.10 2.0 1.30 Killed
			Av. 2.05		Av. 0.66		
Litter 2. 48 hrs. starvation.							
1	662	0.087	7.81	0.28	56	0.59	Control
2	790	0.090	7.86	0.79	209	0.40	"
			Av. 0.53		Av. 0.49		
3	732	0.018	6.28	0.87	216	0.55	11.30 a.m. 1 unit 1.00 p.m. 1 " 2.15 Killed
4	654	"0"	6.00	1.09	242	0.51	11.30 a.m. 1 unit 1.00 p.m. 1 " 2.00 Killed
5	417	"0"	—	0.48	72	0.60	11.30 a.m. 1 unit 1.00 p.m. 1 " 1.15 Killed
			Av. 0.81		Av. 0.55		

that an increase was occurring in liver-glycogen without any attendant decrease in peripheral glycogen. In the publication of Corkill, above referred to, the increase in the liver-glycogen of insulinised young starving rabbits was associated with an average fall in muscle-glycogen and it was calculated that the peripheral loss was at least as great as the gain in the liver. His emphasis on this matter seems to suggest that it was considered that a transference was occurring from the muscles to the liver, an assumption for which there is at present no experimental evidence. In our experiments such large peripheral losses have not been found and the increase in liver-glycogen may certainly occur when no loss at all has been sustained by the muscles.

There seems to be no question that insulin stimulates peripheral oxidation and hence we must consider that in the above experiments and in those on young rabbits and cats, either there was proceeding a simultaneous replenishment of glycogen in the muscles or the effect on oxidation is a later one. Indeed Macleod [1930] has recently suggested that this accumulation of glycogen in the liver after insulin is an early effect of the hormone; but it is

sometimes difficult to accept this because the animal may die in hypoglycaemia with an immensely increased liver-glycogen and a very considerable though diminished muscle-glycogen. In some cases a synthesis of glycogen could be demonstrated in muscle as well as in the liver [Goldblatt, 1930].

It is clear that no theory of the action of insulin can approach the truth which does not take into account that it can produce a fall in blood-sugar in the perfused decapitated eviscerated cat. But this action of the hormone seems to be influenced in the intact animal by the presence of the liver and other mechanisms, which are available to different extents in different species. Further we have no evidence as to the amount of insulin which is normally operative in different species; so that when we superimpose doses of insulin in acute experiments, the normal mechanism or its velocity is probably entirely disturbed.

In the following experiments we propose to show that even in the human subject the effect of insulin on the respiratory metabolism may vary with the conditions.

One of us (M. W. G.) was the subject of experiment. Starvation was for a period of 16 hours before the administration of the insulin. Estimations of metabolism were carried out by the Haldane and Douglas methods. The estimations of the basal values were made after 2 hours' complete rest.

Table IV. *Effect of insulin on the respiratory metabolism of a starving human subject.*

10 units insulin injected subcutaneously at 1.16.

Time	Blood-sugar g. per 100 cc.	Ventilation per min. litres	O ₂ per min. cc.	CO ₂ per min. cc.	Total R.Q.	Non- protein R.Q.
11.20	0.109	—	—	—	—	—
12.05	—	5.66	226	186	0.83	0.84
12.50	—	5.16	206	165	0.80	0.80
1.46	—	4.93	179	145	0.81	0.82
2.05	0.045	—	—	—	—	—
2.10	—	6.78	246	242	0.98	1.01
2.20	0.062	—	—	—	—	—
2.42	—	5.72	200	176	0.88	0.92
2.50	0.067	—	—	—	—	—

At 2 p.m. a moderately severe hypoglycaemic reaction was experienced, apprehension, sweating and hunger being marked. It will be observed that at the time of greatest fall in blood-sugar, the R.Q. had reached unity and the O₂ utilisation had increased very definitely. This increase in oxygen utilisation was the result of the greater ventilation and not of an increased difference in the compositions of the inspired and expired air. The CO₂ difference, however, rose from 3.6 to 3.9, so that it may be considered that there was a true increase in oxidation of carbohydrate with a secondary stimulus to the respiratory centre from the rise in CO₂ production. Recovery was spontaneous and the rise in blood-sugar was accompanied by a fall in R.Q. and total metabolism.

This type of metabolic change after injection of insulin is, in our experience, always reproducible in the starving organism. The apparent drop in protein metabolism is unusual and is quickly balanced by the subsequent rise. Such results give considerable support to the view that insulin provides a stimulus to the oxidation of carbohydrate, but the matter is more complicated than this.

In the following experiments (certain of which were carried out with Dr William Maclean) it will be demonstrated that:

(1) The fall in blood-sugar produced by insulin is not necessarily accompanied by a rise in R.Q.

(2) The rise in R.Q. which follows the injection of insulin in the fasting state can be inhibited by giving glucose simultaneously.

(3) The reduction of blood-sugar to negligible proportions may be accompanied by an acute fall in R.Q.

These experiments were performed on diabetic patients and on glycosurics without ketosis.

The effect of insulin on a glycosuric without ketosis.

The patient was a woman of 50 years and 15 st. weight. In Exp. A a light meal was taken at 8.30 a.m. In B starvation for 16 hours before the experiment.

	Time	Blood-sugar g. per 100 cc.	R.Q.	O ₂ per min. cc.	Urine-sugar g. per 100 cc.
Exp. A	10.56	0.304	0.89	275	3.80
	11.08	10 units insulin subcutaneously			
	11.35	0.328	0.85	286	—
	12.05	0.286	0.84	271	2.74
	1.05	0.230	0.83	255	0.41
	2.05	0.200	0.81	258	0
	2.38	0.181	0.85	252	0
	4.20	0.130	0.83	244	0
	5.30	0.117	—	—	—
Exp. B	11.25	0.348	0.74	304	3.34
	12.25	5 units insulin subcutaneously			
	12.55	0.301	0.68	296	2.00
	1.35	0.275	0.77	262	1.76
	2.15	0.240	0.72	281	0.66
	3.00	0.212	0.76	272	0.18
	4.26	0.177	0.75	261	0

The subject was a woman of active life (a washerwoman) and complained only of obesity. We were unable by any therapeutic measures to influence her obesity, partly because she did not reduce her intake of food. She was losing regularly about 100 g. sugar per day in the urine. Her threshold for sugar was definitely raised, as can readily be seen above.

It is clear from the above figures that the prevention of the glycosuria was not accompanied by any change in the degree or type of metabolism, so that we must suppose an inhibition of release of sugar from the liver to be taking place under the action of the hormone. It is important to note that this type of patient rarely shows evidence of ketosis and, in fact, in this case there was never any excretion of acetone which could be estimated. The conditions

usually taken as evidence of gluconeogenesis were absent in spite of the great loss of sugar and the high fasting blood-sugar.

It thus appears that a steady fall in blood-sugar may be induced without any observable change in metabolism. Of course the doses were small and give no information of what may occur in severe hypoglycaemia. In the next experiments very much larger amounts of insulin were used and very low levels of blood-sugar were obtained; the subjects were diabetics.

The effect of insulin on diabetic subjects.

Case 1. A young woman of 19 years. Very severe diabetic with all the classical symptoms. Always strong ketosis present. Before each experiment food was stopped for 16 hours and insulin not injected after the morning dose of the previous day.

We first give the reaction of this subject to glucose and fructose.

				Urine-sugar	
Time after sugar (hrs.)	Blood-sugar g. per 100 cc.	R.Q.	O ₂ per min. cc.	Total during experiment	
				g. per 100 cc.	g.
Effect of 50 g. glucose <i>per os</i> .					
0	0.286	0.79	260	3.3	
$\frac{1}{2}$	0.390	0.71	264	6.1	
1	0.420	0.74	281	6.6	
$1\frac{1}{2}$	0.410	0.75	259	8.5	
2	—	0.74	248	7.1	43.31
Effect of 50 g. fructose.					
0	0.373	0.69	227	7.25	
$\frac{1}{2}$	0.403	0.80	232	6.42	
1	0.423	0.68	259	7.25	
$1\frac{1}{2}$	0.443	0.76	233	7.62	
2	0.414	0.78	219	7.75	40.2

These results present the classical features of a very severe diabetic. A certain amount of support is given by these findings to the view that the diabetic organism can make somewhat better use of fructose than of glucose.

In the following two experiments the effect of insulin was examined.

Time after insulin (hrs.)	Blood-sugar g. per 100 cc.	R.Q.	O ₂ per min. cc.	Urine-sugar	
				g. per 100 cc.	Total g.
Effect of 50 units insulin (subcutaneously) followed 1½ hours later by 50 g. fructose.					
0	0.343	0.77	209	8.30	
0	---	0.74	235	—	
1	0.252	0.75	215	6.6	
1½	0.209	0.91	211	6.05	33.5
50 g. fructose <i>per os</i> .					
2	0.187	0.88	227	2.80	
2½	0.206	0.85	235	1.50	
3	0.166	0.73	227	1.03	
3½	0.162	0.80	236	0.50	0.98
Effect of 50 units insulin and 50 g. glucose <i>per os</i> at same time.					
0	0.187	0.76	258	—	
½	0.388	0.68	257	6.60	
1	0.398	0.72	241	7.10	
1½	0.353	0.76	238	8.50	
2	0.302	0.72	229	9.00	
3	0.209	0.73	237	5.30	
4½	0.104	0.76	235	0.85	34.64

The peculiar fact emerges from these findings that whereas insulin produced a rapid rise in R.Q. whilst the patient was starving, this effect was either diminished by giving fructose or completely inhibited if glucose were given simultaneously with the insulin (of course this is not specific). Further no significant change in the respiratory metabolism occurred even when the blood-sugar reached normal levels in the latter case.

Thus again it seems that insulin may promote a synthetic action without any simultaneous change in the oxidative processes.

In the following case similar observations were made.

Case 2. Patient was a girl of 14 years. Severe diabetic. Experiments were performed 16 hours after food and insulin.

Time after sugar (hrs.)	Blood-sugar g. per 100 cc.	R.Q.	O ₂ per min. cc.	Urine-sugar	
				g. per 100 cc.	Total g.
Effect of 30 g. glucose <i>per os</i> .					
0	0.420	0.69	215	5.65	
$\frac{1}{2}$	0.505	0.72	208	6.30	
1	0.465	0.77	201	6.82	
$1\frac{1}{2}$	0.408	0.83	200	7.10	
2	0.371	0.75	204	6.82	34.7
Effect of 30 g. fructose <i>per os</i> .					
0	0.353	0.78	183	4.75	
$\frac{1}{2}$	0.420	0.86	186	5.12	
1	0.381	0.76	206	6.38	
$1\frac{1}{2}$	0.383	0.88	197	6.56	
2	0.381	0.93	189	6.38	28.0

In this case we again see some evidence that the diabetic may make a more successful use of fructose than of glucose.

The findings in this case serve to show how various may be the reactions

Time after insulin (hrs.)	Blood-sugar g. per 100 cc.	R.Q.	O ₂ per min. cc.	Urine-sugar	
				g. per 100 cc.	Total g.
Effect of 50 units insulin followed by 30 g. fructose 1½ hours later.					
0	0.388	0.78	142	7.10	
1	0.201	0.96	155	5.40	
1½	0.133	0.84	178	1.03	35.0
30 g. fructose <i>per os</i> .					
2	0.067	0.85	162	0.30	
2½	0.022	0.77	205	0	0.30
The very low blood-sugar reached at this time made it necessary to give some carbol 50 g. glucose were accordingly given <i>per os</i> .					
3	0.090	0.90	204	0	0
Effect of 50 units insulin and 30 g. glucose 1½ hours later.					
0	0.374	0.75	201	5.65	
½	0.338	0.77	—	5.25	
1	0.302	0.78	204	5.77	
1½	0.333	0.79	198	6.30	30.0
30 g. glucose <i>per os</i> .					
2	0.328	0.82	204	6.17	
2½	0.339	0.84	202	7.35	
3	0.344	0.78	192	8.17	
3½	0.296	0.76	201	7.76	14.6

of the respiratory metabolism to insulin even in the same subject. For whereas on one occasion 50 units produced an acute rise in R.Q. to 0.96 with a fall in blood-sugar from 0.388 to 0.201, on another occasion the same dosage and conditions only raised the R.Q. from 0.75 to 0.78 and the blood-sugar fell only from 0.374 to 0.302.

Very striking was the sharp drop in R.Q. (0.77) which occurred when the blood-sugar fell to 0.022. We have observed this on several occasions and can only attribute it to a retention of CO_2 in the tissues as a result of some unknown factor. There is of course the possibility that at so low a level of glycaemia there may be so great a deficiency of circulating carbohydrate that other substances are brought into the metabolic field, thus lowering the R.Q. As we have reason to believe that these substances cannot be protein, they might possibly be fat, a view which would be in harmony with the increased excretion of ketones which occurs during insulinisation of fat-fed rats.

Attention may be drawn here to the fact that it has been found that in hypoglycaemia the venous blood is highly oxygenated [Klein, 1930; Klein and Holzer, 1929] and approximates in its O_2 content to arterial blood. The arterio-venous CO_2 difference was also found by these authors to be very much diminished. They conclude that the O_2 utilisation by the tissues in insulin shock is very greatly diminished. This is, however, a conclusion which seems to us quite untenable, seeing that estimations of respiratory metabolism will almost always demonstrate a greatly increased utilisation of O_2 and especially in hypoglycaemia. If through some interference with capillary function the removal of CO_2 were interfered with, then the sudden fall in R.Q. might be accounted for. In the following experiment we show another example of the same phenomenon of fall in R.Q. in extreme hypoglycaemia.

Case 3. The patient was a mildly diabetic man. All experiments carried out after 16 hours without food or insulin.

Time after sugar (hrs.)	Blood-sugar g. per 100 cc.	R.Q.	O ₂ per min. cc.	Urine-sugar	
				g. per 100 cc.	Total g.
Effect of 50 g. glucose <i>per os</i> .					
0	0.146	0.76	208	0.20	
$\frac{1}{2}$	0.171	0.74	224	0.20	
1	0.206	0.95	194	0.85	
$1\frac{1}{2}$	0.241	0.89	185	2.15	
2	0.246	0.87	197	2.45	3.51
Effect of 50 g. fructose.					
0	0.241	0.84	244	1.95	
$\frac{1}{2}$	0.261	0.87	257	1.50	
1	0.281	0.84	238	1.95	
$1\frac{1}{2}$	0.277	0.85	275	2.07	
2	0.271	0.85	297	2.70	15.16
Effect of 25 units insulin and 50 g. glucose at same time.					
0	0.129	0.73	209	0.24	
$\frac{1}{2}$	0.146	0.72	240	0.25	
1	0.206	0.83	209	1.25	
$1\frac{1}{2}$	0.231	0.82	209	2.05	
2	0.222	0.83	211	1.64	
$2\frac{1}{2}$	0.166	0.80	204	0.95	3.54

Effect of 20 units insulin followed 1 hour later by 50 g. fructose *per os*.

Time after insulin (hrs.)	Blood-sugar g. per 100 cc.	R.Q.	O ₂ per min. cc.	Urine-sugar	
				g. per 100 cc.	Total g.
0	0.156	0.74	242	0.70	
$\frac{1}{2}$	0.166	0.76	252	0.37	
1	0.150	0.84	239	0.27	2.80
	50 g. fructose <i>per os</i> .				
$1\frac{1}{2}$	0.109	0.92	254	0.25	
2	0.104	0.92	272	0.34	
$2\frac{1}{2}$	0.060	0.91	277	0.26	
3	"0"	0.79	271	0	0.60

Thus it is seen that the administration of glucose simultaneously with the insulin did not produce as great a change in R.Q. as when the glucose was given alone.

Again we find that very low levels of blood-sugar (in this case the symbol "0" indicates that the thiosulphate equivalent of the Cu-reducing substances in 0.2 cc. blood is less than 0.2 cc. ($N/400$)) were associated with an extraordinarily rapid fall in R.Q. not accompanied by any change in oxygen utilisation and in spite of the large amount of available sugar.

In case 2 the sudden fall of R.Q. at the blood-sugar level of 0.022 was associated with a large rise in oxygen absorption (43 cc. per min.) but the increase in CO₂ output (20 cc. per min.) was not sufficient to keep the R.Q. up. In case 3 just considered there was no rise in oxygen utilisation at the critically low blood-sugar but the CO₂ output suddenly fell. It seems to us highly improbable that this sudden change in R.Q. is due to a change in type of metabolism and we consider it much more likely that we are dealing with some retention of CO₂ not at present explicable. Such low levels of blood-sugar as we have reached have not, to our knowledge, been reported in the literature in association with estimations of respiratory metabolism and as it is not often that one dares, in the human subject, continue to such low levels, the phenomenon may be readily overlooked.

Results of such a kind raise the old difficulty which assails every attempt to interpret respiratory quotients. However, from the above results we think that the conclusion may be drawn that insulin may bring about a definite stimulus to carbohydrate oxidation but that this is not the only mechanism by which the blood-sugar is lowered, since the effect may quite certainly be produced without any perceptible change in respiratory metabolism.

A complete knowledge of the action of insulin must involve the power to foretell exactly what will occur in given conditions. It must be admitted that we cannot as yet do this. In one condition, however, *viz.* in starvation, insulin seems always to produce an increase in R.Q. but not necessarily a rise in heat production. When the conditions are made more complicated, as by administration of carbohydrate or the production of extreme hypoglycaemia, it appears that secondary influences enter which make it difficult to interpret the findings of respiratory metabolism. Where, however, in the presence of

large amounts of sugar, the R.Q. does not change after the administration of insulin in large doses, it seems reasonable to accept glycogen synthesis as the most probable explanation.

Further experiments on the effect of insulin on nitrogen excretion.

In certain of the above experiments the effect of insulin on the protein metabolism of rats in starvation was examined and it was found that no appreciable effect occurred. In the following series an attempt was made to see if the injection of the hormone during periods of exclusively fatty diets could modify the excretion of nitrogen.

Ketones were estimated by a method described by one of us [Goldblatt, 1925]. The animals were albino rats bred in the laboratory and kept previously to the experimental period on a diet of bran, oats and occasionally green stuff.

Exp. 1. An adult rat was starved during 3 days and treated as below.

Day	Wt. g.	Urine		Remarks
		Total N g. per 100 g. initial wt.	Total ketones as mg. acetone	
1	207	0.072	1.10	Starvation
2	197	0.063	0.80	Starvation
3	185	0.058	0.80	Starvation and 0.4 unit insulin at 2.30 p.m. and 4.50 p.m.

Clearly no significant change in either nitrogen or ketones occurred.

Exp. 2. This experiment was carried out during the summer, which will explain the type of ketone excretion which occurred. An adult rat was taken and put on a diet of lard and 5% salt mixture (Wigglesworth) and water. Treatment as below.

1	163	0.047	0.80	Fatty diet commenced
2	160	0.068	3.20	
3	154	0.040	21.00	
4	152	0.042	4.50	
5	152	0.027	0.80	Adaptation complete
6	152	0.040	2.50	2 doses 0.4 unit insulin
7	158	0.046	1.10	1 dose 0.8 unit insulin

Exp. 3. The same rat and the same diet as in the last experiment.

1	---	0.039	1.00	Fat diet commenced
2	---	0.075	2.40	
3	---	0.080	17.50	
4	---	0.053	10.90	
5	---	0.045	8.60	
6	}	0.065	8.20	Adaptation almost complete
7				
8	---	0.038	3.10	2 doses 0.4 unit insulin
9	---	0.032	1.10	2 doses 0.4 unit. Animal weak and hypoglycaemic about 2 hours after the second injection. No convulsions
10	---	0.040	0.90	

On neither diet was the nitrogenous excretion modified by insulin. Excretion of ketones was increased by insulin in the rats on the fatty diet in the summer [cf. Burn and Ling, 1928].

In the next experiment similar estimations were made with the addition

that the period was extended, the exact amount of fat eaten per day was determined and rather greater doses of insulin were administered.

Exp. 4. Two rats were used in the same cage. The fat diet was the same as that used in the previous cases. The diet was fed by hand, so that the exact amount eaten per day was known.

Urine					Remarks
Day	Wt. g. (combined)	Fat eaten g.	N per 100 g. animal g.	Ketones as mg. acetone	
1	287	0	0.074	1.30	Starvation
2	259	17.53	0.092	1.60	
3	266	17.95	0.061	2.00	
4	270	8.92	0.040	2.20	Ketosis of winter type
5	266	3.02	0.041	1.20	
6	254	4.97	0.048	0.80	Adaptation complete
7	245	7.32	0.047	2.10	0.28 g. NaHCO_3 taken by animals from a solution put in place of water
8	242	19.96	0.042	3.50	0.24 g. NaHCO_3
9	237	6.12	0.037	1.90	Each 3 doses of 0.3 unit insulin—no symptom
10	—	8.14	—	6.30	Each 0.5 unit—both hypoglycaemic
11	226	9.20	0.038	2.10	No treatment
12	225	7.36	0.049	3.90	Each 0.5 unit
Total		110.49	1.549	29.00	

The total fat excreted in the faeces was <2 g.

This experiment demonstrates clearly the existence of a basal nitrogen excretion on a nitrogen-free diet, in this case about 0.04 g. N per 100 g. body weight. Further this quantity is independent of the actual amount of fat ingested daily. The insignificant excretion of ketones confirms the findings of other workers, who find that the commonly held views on ketosis are in no sense applicable to the rat.

The changes in the excretion of nitrogen after insulin were clearly of no significance and again confirm the findings in the previous experiments. It will also be seen that the excretion of ketones was, after adaptation was complete, increased to a greater extent by insulin than by NaHCO_3 administered under similar conditions. These sudden rises in ketosis after insulin are not readily explicable. The view taken by Hirschhorn and Pollak [1927] as to the action of adrenaline on ketosis may be partly applicable to that of insulin, viz. that there occurs a sudden drain on the hepatic glycogen and that it is the suddenness of this drain which determines the degree of increase of ketosis. On this view a quantitatively equal loss of glycogen more gradually released would be followed by a smaller degree of ketosis.

The interesting fact that rats can continue for so long as in the above experiments in health and relatively slow rate of loss in weight on a diet consisting solely of fat and salts raises the contested question as to whether fat can give rise to carbohydrate. There can be no doubt that rats killed after prolonged periods of exclusively fatty feeding show very considerable quantities of glycogen in the liver and tissues. This matter will however be dealt with in a further communication.

CONCLUSIONS.

From these experiments it appears justifiable to consider that, in the normal animal, starving or consuming a diet containing no nitrogen, there is no change in the excretion of nitrogen as a result of the injection of insulin. The effect of insulin therefore appears to be limited to the metabolism of fat or carbohydrate or both.

The effect of insulin on peripheral oxidation is not one which, as far as our evidence goes, can exert any profound influence on the general total metabolism, as was seen in our growth experiments with rats. Nor is an increase in oxidative processes a necessary accompaniment of the fall in blood-sugar which follows the injection of the hormone.

SUMMARY.

1. Litters of white rats, kept on restricted or unrestricted amounts of food, showed no difference in rate of growth from that of litter mates, when injected with daily doses of insulin varying from 2 to 50 units.

2. Insulin was found to have no effect on the nitrogen excretion in the urine of rats consuming an exclusively fatty diet or during starvation. This was the case whether hypoglycaemia did or did not occur; convulsions were absent.

3. Insulin hypoglycaemia in a starving human subject is associated with a marked increase in R.Q. and hence presumably an increased carbohydrate- and diminished fat-oxidation. This effect passes over as the blood-sugar recovers.

4. Progressive fall in blood-sugar and disappearance of glycosuria may be induced in a glycosuric patient without any change in R.Q. or calorie output. It is considered that this must indicate glycogen synthesis in the liver.

5. Insulin can increase the liver-glycogen of young dogs during starvation without any change in muscle-glycogen. This effect may be accompanied by profound hypoglycaemia.

6. The attainment of blood-sugar values of vanishing proportions may be accompanied by an acute fall in R.Q. It is considered improbable that this fall indicates a true change in metabolism.

We wish to thank Professor Hugh MacLean of this Unit for his constant advice and encouragement.

REFERENCES.

- Burn and Ling (1928). *J. Physiol.* **65**, 191.
Chaikoff and Macleod (1927). *J. Biol. Chem.* **73**, 725.
Corkill (1930). *Biochem. J.* **24**, 779.
Goldblatt (1925). *Biochem. J.* **19**, 626.
—— (1930). *Biochem. J.* **24**, 1199.
Hirschhorn and Pollak (1927). *Z. klin. Med.* **105**, 371.
Klein (1930). *Z. ges. exp. Med.* **73**, 787.
—— and Holzer (1929). *Z. ges. exp. Med.* **64**, 798.
Long and Bischoff (1930). *J. Nutrition*, **2**, 245.
Lublin (1926). *Arch. exp. Path. Pharm.* **115**, 101.
Macleod (1930). *Lancet*, ii, 512.
——, Magee and Middleton (1930). *Biochem. J.* **24**, 615.

XXX. THE DISTRIBUTION OF NITROGEN AND SULPHUR IN THE URINE DURING CONDITIONS OF INCREASED CATABOLISM.

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As the result of a series of analyses of the urine of normal subjects on standard diets of varying nitrogen and sulphur content but practically free of purine, creatine and creatinine, Folin [1905] advanced the hypothesis that there are two essentially different forms of protein catabolism, the constant or endogenous catabolism yielding chiefly creatinine and neutral sulphur and to a less extent uric acid and ethereal sulphates, and the variable catabolism yielding chiefly urea and inorganic sulphates. Folin stated that "the more the total catabolism is reduced the more prominent become these representatives of the constant catabolism, and the less prominent become the two representatives of the variable catabolism."

Whilst Folin's general hypothesis has been accepted, doubt has been expressed as to whether these various excretory products can be exactly divided into two such groups.

More recent work has demonstrated the difficulty of correlating the creatinine output with the total tissue or endogenous metabolism. Shaffer [1907; 1908, 2], Spriggs [1907, 1, 2], Paton [1910] and Benedict and Osterberg [1923] consider that creatinine is an index of only one phase of this tissue metabolism—that which is peculiarly related to muscle. Spriggs also concluded from his observations that the bulk of the endogenous creatinine output is "a product of the internal structural metabolism of muscle and not of its contraction." Some 30 years ago Sivéń [1901] and Burian and Schur [1901] showed severally that uric acid is to a large extent independent of the total amount of nitrogen eliminated. Folin states that while uric acid does not exhibit the quantitative constancy that is found in the case of creatinine at least a certain part of it represents the breaking down of living protoplasm which must be supposed to be essential to the continuation of life. The reduction in the uric acid output when the total metabolism of protein is reduced is irregular and different for different individuals. Pohl [1917] has more recently suggested that uric acid might be included as a principal endogenous catabolite.

Practically all experiments bearing on this aspect of metabolism have been concerned with studying the effect of different levels of food intake on the partition of the nitrogen- and sulphur-containing catabolites of the urine. Starvation may be taken as an extreme example of this type of experiment. Benedict [1907] found that preformed creatinine and neutral sulphur decreased persistently during fasting. The interesting point arises however as to the effect of increased catabolism on the distribution of the nitrogen and sulphur of the urine where the food intake is kept constant. The main causes generally accepted for such increased protein catabolism are increased muscular work, disuse of muscle, injuries to and toxic conditions of the tissues including febrile states, exophthalmic goitre and various other wasting diseases.

Gregor [1900] concluded that hard physical exercise produces a decided increase in creatinine elimination particularly on the day following the exercise. Campbell and Webster [1921, 1922] and Rakestraw [1921] have also noted an increased output.

While Shaffer [1908, 1] found that muscular work had no influence on the excretion of uric acid, Kocher [1914], Rakestraw [1921] and Levine *et al.* [1924], have since found that it caused a slight increase. Others like Dunlop *et al.* [1897], Campbell and Webster [1921, 1922] and Hartmann [1924] however record a decrease.

While Shaffer [1908, 1] and Campbell and Webster [1921, 1922] have found that work exercises but little influence on the output of ammonia, Dunlop *et al.* [1897] have observed a rise, and Kocher [1914] a definite reduction (nitrogen-free diet) in the output of this catabolite.

In more recent carefully controlled experiments on the human subject, Cathcart and Burnett [1926] have detected a slight but constant rise in the urinary excretion of nitrogen and sulphur after work. This was accompanied by a slight uniform rise in the excretion of ammonia, creatinine and urea, although the latter was not directly analysed. Dunlop *et al.* [1897], Shaffer [1908, 1], Kocher [1914] and Campbell and Webster [1921, 1922] have also found that for the most part the definite rise in the nitrogen after work is in the form of urea.

The present writer [Cuthbertson, 1929], found that subjects in nitrogenous equilibrium showed within a day or two from the commencement of a period of rest a rise in the excretion of sulphur, nitrogen, phosphorus and calcium. The S:N ratio suggested a sulphur-rich source of the excreted material. It was found that the rise in the excretion of sulphur was due to a slightly greater proportionate increase in the inorganic sulphate fraction. Ethereal sulphates tended to decrease; neutral sulphur remained more constant. The rise in the excretion of total nitrogen was mainly due to a proportionate increase in the amount of urea. Ammonia tended also to rise proportionately while the absolute amounts of creatinine and uric acid remained practically unaltered. A very slight rise in the excretion of creatinine was sometimes noted, particularly as the period of enforced rest lengthened.

In various febrile conditions, it has been found that on the average 82 % of the total nitrogen in the urine is in the form of urea [von Noorden, 1907]. Ammonia excretion is also increased considerably, but the percentage increase is generally not greater than the percentage increase in the total nitrogen. The endogenous output of uric acid is also increased, the increase coinciding and terminating with the fever [Cathcart, Kennaway and Leathes, 1908]. Although the output of creatinine is almost always increased in febrile conditions the rise is not proportional to the increased elimination of total nitrogen [Leathes, 1906; Shaffer, 1908, 2]. Leathes [1906] for example found that while there was a 50 % increase in the total nitrogen, there was only a 20 % increase in the creatinine nitrogen in one particular case. In the case of uric acid the proportion excreted was considerably increased.

Sandiford *et al.* [1926] found that the injection of thyroxine on the 30th day of a protein-free diet in the human subject produced a subsequent rise in the excretion of nitrogen mainly in the form of urea, creatinine and uric acid remaining constant. Shaffer [1907 and 1908, 2] had many years ago noted that the amount of creatinine excreted in exophthalmic goitre was very low, despite the increased tissue catabolism.

The purpose of the short foregoing review has been to demonstrate that in conditions where the nitrogen and sulphur reserves of the body are being catabolised to any great extent and where there is little or no change in the food intake the main increase in the urinary excretion is accounted for as urea. The proportion of this catabolite eliminated may even be slightly greater. Creatinine on the other hand exhibits practically no change in the absolute amount excreted. Occasionally when the increased catabolism is at a high level this substance may be excreted in slightly greater amount, but it is never increased proportionately. The excretion of uric acid, while not so constant as that of creatinine, is relatively stable, such fluctuations as occur are usually in the form of a rise. It may be that there is a special metabolism of nucleoprotein. Ammonia generally tends to rise with the increased catabolic level.

Concerning sulphur not so much is known. It may be generally stated that a rise in total sulphur is due to a practically proportionate increase in the inorganic sulphates. Neutral sulphur remains practically constant. The ethereal sulphates may diminish.

Such findings as have been described appear in agreement with the laws governing the composition of the urine as enunciated by Folin [1905].

More recently the present author [Cuthbertson, 1930] has investigated, in part, the effect of tissue injury on metabolism. It was found that the urinary excretion of sulphur, nitrogen and phosphorus rose rapidly to a maximum generally within 3 to 6 days from the time of injury. The S:N ratio suggested that some sulphur-rich tissue, such as muscle, might be the main source of the material catabolised. The partitions of the nitrogen and sulphur were not determined at that time. They are amplified in the present investigation.

Table I. *The partition of nitrogen and sulphur in the urine of patients at rest in bed during their recovery from fractures of bone or minor operations.*

Case	Total daily excretion of nitrogen g.	Daily excretion and (partition) of nitrogen					Total daily intake of N g.	Daily excretion and (partition) of sulphur (SO ₃)				Remarks	
		Urea	Ammonia	Amino-acid	Total creatinine	Pre-formed creatinine		Uric acid	Total	Inorganic	Ethereal		Neutral
1	32.06*	26.09 (81.4)	1.56 (4.9)	1.14 (3.5)	1.29 (4.0)	— (0.6)	0.195 (0.6)	11.70	4.96 (83.1)	4.12 (83.1)	0.24 (4.8)	0.60 (12.1)	Fourth day following a fracture of both bones of one leg of a boy aged 16 years. Average daily output of faecal nitrogen 1.51 g.
2	9.29	7.69 (82.8)	0.20 (2.2)	0.06 (0.7)	0.51 (5.5)	0.45 (4.8)	0.18 (1.9)	9.32	1.73 (72.5)	1.255 (72.5)	0.155 (9.0)	0.32 (18.5)	Prior to operative removal of torn cartilage from knee joint of man. Third day after operation
3	7.66	6.41 (83.7)	0.35 (4.6)	0.04 (0.5)	0.40 (5.2)	0.39 (5.1)	0.09 (1.2)	7.06	1.79 (74.9)	1.34 (74.9)	0.09 (5.0)	0.36 (20.1)	Fourth day after fracture of both bones of one leg of a man. Seventh day after
4	12.70*	11.44 (81.0)	0.28 (2.2)	0.03 (0.2)	0.42 (3.3)	0.41 (3.2)	0.18 (1.4)	7.06	2.30 (77.8)	1.79 (77.8)	0.14 (6.1)	0.37 (16.1)	Prior to operative removal of torn cartilage from knee joint of man. Third day after operation
5	16.90*	13.49 (79.8)	0.495 (2.9)	—	0.47 (2.8)	0.475 (2.5)	0.14 (0.83)	8.36	2.57 (84.05)	2.16 (84.05)	0.17 (6.6)	0.24 (9.35)	Eighth day after fracture of the femur of a man
6	10.08	8.51 (84.4)	0.515 (5.1)	0.03 (0.3)	0.58 (5.7)	0.55 (5.4)	0.10 (1.0)	12.67	2.14 (77.1)	1.65 (77.1)	—	—	Third day after a dislocation of the ankle of a man. Sixth day after
7	19.74*	17.24 (87.3)	0.45 (2.3)	0.22 (1.1)	0.70 (3.5)	0.70 (3.5)	0.23 (1.2)	12.67	3.29 (86.6)	2.85 (86.6)	—	—	Third day after fracture of tibia of a man. Sixth day after
8	11.86	9.85 (73.8)	0.90 (6.8)	0.16 (1.2)	0.57 (4.3)	0.57 (4.3)	0.17 (1.3)	10.77	2.79 (80.0)	2.23 (80.0)	0.15 (5.3)	0.41 (14.7)	Prior to bone-grafting operation for ununited fracture of humerus of 6 years standing in a man. Fourth day after operation
9	20.58*	18.45 (89.6)	0.66 (3.2)	0.31 (1.5)	0.68 (3.3)	0.625 (3.0)	0.26 (1.2)	10.77	3.81 (80.6)	3.07 (80.6)	0.19 (5.0)	0.53 (14.4)	Prior to bone-grafting operation for ununited fracture of humerus of 6 years standing in a man. Fourth day after operation
10	17.53*	15.05 (85.8)	0.91 (5.2)	—	0.63 (3.7)	0.36 (2.0)	0.25 (1.5)	12.10	2.97 (85.9)	2.55 (85.9)	0.15 (7.3)	0.37 (18.0)	Prior to bone-grafting operation for ununited fracture of humerus of 6 years standing in a man. Fourth day after operation

* Maximum daily excretion of nitrogen.
The excretion of urinary nitrogen was determined daily throughout the periods of observation, but these values are omitted except on the days on which the distribution was detailed.
Methods. Total N, Kjeldahl; urea, urease; ammonia, aeration; creatine and creatinine, Folin; amino-acids, Sørensen; uric acid, Hopkins; total sulphur, Denis; inorganic and ethereal sulphates, Folin.
The figures for excretion are g.; those for partition %.

EXPERIMENTAL.

The subjects were patients at rest in bed during recovery from fractures of bone or minor operations and generally within 1 or 2 days following the injury were put on a constant intake of food, the quality and quantity of which they chose from the following list: milk, brown and white bread, digestive biscuits, butter, eggs (with the exception of case 5), tea (2 teaspoonsfull infused daily: no tea in cases 5, 7 and 8), steak (except in cases 3, 4, 5, 7 and 8), potatoes, lettuce, apple and orange. As they had been on a normal diet prior to admission, it was thought that the nitrogenous intake would be closer to this normal if a meat-containing diet was consumed. Subsequently meat-free diets were selected in order to assess the endogenous metabolism. The analytical results are summarised in Table I.

Nitrogen.

Total nitrogen. The maximum total daily output of urinary nitrogen of this series occurs between the 3rd and 8th days following the injury. There does not appear to be any definite relationship between the time interval for the urinary excretion of nitrogen to reach a maximum and the total amount passed on that particular day. The maximum daily loss may even exceed 20 g. (Case 1).

Urea. There is an absolute increase in the amount of urea, which increase is practically proportional to the raised urinary output of total nitrogen (81–82 %).

Ammonia. The absolute amount of ammonia tends to vary but there is generally a relative diminution in the proportion of the total nitrogen excreted in this form.

Amino-acids. There is generally a rise in the absolute amount passed (as measured by the nitrogen content) and this rise is practically proportional to the rise in total nitrogen.

Creatinine (preformed). Except for slight increases in cases 6 and 7 there is little or no alteration in the absolute amount passed.

Uric acid. There is generally an absolute rise in the excretion of uric acid, but this rise may or may not be proportional to the increased output of total nitrogen.

Creatine. A creatinuria with a normal (or even supernormal) output of creatinine has been noted following amputations of limbs [Bürger, 1919; Cameron and Gibson, 1922], and also sometimes after fractures, particularly of the thigh or leg bones. Hirst and Imrie [1928] found that in such fractures the amount varied from traces to over 500 mg. daily. It disappeared gradually during the healing. Administration of thyroid increased the output threefold.

In cases 2, 4 and 8 only was a pre-injury period available. Case 2 was on a meat diet and cases 4 and 8 on a non-meat diet. It is seen that whereas in cases 2 and 4 the total creatinine was constant during the pre-operative

and post-operative periods there was in case 2 in the post-operative period of maximum catabolism a slight increase in the creatine fraction and in case 4 there was only a trace of creatine on the 3rd day following the operation. Prior to operation case 8 had no creatinuria, but on the day after operation there was a very definite amount present, the creatinine fraction being slightly diminished. Case 3, who was also on a meat-free diet, showed no change in the total creatinine excretion nor was any creatine present. No creatinuria was found at the height of catabolism in case 6 although on the 3rd day after admission there was a trace present. This case had a dislocated ankle but there was no fracture or surface wound present. In case 7 no creatine was present shortly after admission but on the day of the appearance of the maximum excretion of nitrogen a trace was found. It appears then that a temporary creatinuria may follow injury. It may be that this is due to an interference with the normal transformation of creatine into creatinine, such as appears to occur in conditions of heightened catabolism.

Proteinuria. On the 3rd day following injury in case 1 there was a faint trace of coagulable protein; on the 4th day there was a definite trace (day of appearance of maximum nitrogen excretion); on the 5th day a faint trace, on the 6th day a very faint trace, thereafter it was absent. In case 4 a trace was noted on the 2nd day following operation only. No evidence of a proteinuria was noted in the other cases described here, although unsystematised testing had often revealed faint traces in cases of recent fracture admitted to hospital.

Sulphur.

In a previous investigation on the disturbance of metabolism produced by injury, it was found that the maximum daily output of sulphur practically always ran parallel with that of nitrogen. As the result of this finding day-to-day determinations of the total nitrogen only were performed and when the maximum excretion was noted the partitions both of nitrogen and of sulphur were determined on that day and also on an earlier date, *i.e.* prior to the appearance of the obvious increase in catabolism. A marked increase in the excretion of sulphur was again noted to follow injury.

Inorganic sulphate. Not only is there an absolute increase in the amount of inorganic sulphate excreted but the percentage of sulphur excreted in this form is greater.

Ethereal sulphate. While there is generally a rise in the excretion of this fraction during periods of increased catabolism, this rise is not proportional to the increase in catabolism as measured by the increase in total sulphur.

Neutral sulphur. There is not quite the same constancy about this fraction as there is about creatinine, but in only one case (7) was there really a pronounced alteration in the amount eliminated as the total sulphur excretion increased. It is of interest that it was in this particular case that a marked increase in the creatinine excretion was noted. As a consequence of this

relative constancy there is a decrease, during periods of heightened catabolism, of that fraction of the total sulphur represented by neutral sulphur.

DISCUSSION.

While these experiments are totally different in kind from those devised by Folin, yet they have an intimate bearing on the two conceptions of metabolism which he describes. It is obvious in the first place that the source of these nitrogen and sulphur losses cannot be the food intake. They must come from the increased catabolic changes in the tissue cells and body fluids. It must also be remembered that it is really only one phase of the total catabolism which has been examined, namely that particularly related to protein.

As the result of the foregoing analyses, there appear to be two distinct forms of metabolism, one constant the other variable. The constant element may exhibit small fluctuations, for example, it may increase with any general increase in catabolism. It is obvious that whilst creatinine and neutral sulphur do chiefly represent the constant catabolism they are not the main representatives of the endogenous catabolism. Urea and inorganic sulphate are the chief products of the increased endogenous catabolism. It seems necessary therefore to dissociate Folin's terms "constant" and "endogenous", "variable" and "exogenous" when describing these and other allied conditions of increased catabolism, for fluctuations in both endogenous and exogenous metabolism are mainly characterised by fluctuations in the variable group of catabolites.

The work of Morpurgo [1897], Greene [1919], Mandel [1928] and others suggests that muscle has a very considerable capacity either for storage or depletion of protein without seriously damaging the life of the individual cell.

It is suggested here that the disintegration and integration of muscle cells through wear and tear and indeed of the body generally, may be peculiarly represented by creatinine and perhaps by neutral sulphur. This does not mean that they are the sole nitrogen- and sulphur-containing catabolites of this form of metabolism. Fluctuations in the urea and inorganic sulphate fractions, although they represent mainly variations in the improvement or impoverishment quotas, may also be an index of changes in the processes of wear and tear. Ammonia is rather an index of the acid-base balance and as such is subject to pronounced variations. Alterations in the excretion of amino-acids and ethereal sulphates are difficult to interpret. Uric acid, although it appears to fluctuate to some extent with the general increase in the total nitrogen excretion, may also be taken to be an index of wear and tear. Possibly it is a catabolic product of the nuclear part of the cell.

The question arises as to the cause or causes of these various catabolic phenomena. Wasting of tissue, particularly of muscle, may be due to an inadequate supply of normal stimuli, or to an excessive number of abnormal stimuli. It may be due to direct poisoning of the tissue cells such as might be supposed to take place in febrile conditions and in tissue injury. The wasting

on the other hand may represent the response to the demand for a non-nitrogenous residue for energy purposes, or it may be part of the mechanism for the rapid production of repair material to heal an injured part. In the present state of our knowledge it is impossible to define the relative significance of these processes.

SUMMARY.

1. The present series of experiments provides additional evidence of the early catabolic loss of nitrogen and sulphur which results from tissue injury. The rate of loss reached a maximum about the 3rd to 8th day following the injury. The maximum daily loss of nitrogen might even exceed 20 g.

2. The partition of the nitrogen- and sulphur-containing catabolites demonstrated that the increase in nitrogen was due to a practically proportionate increase in the urea excreted, and that the increase in sulphur was due to a slightly greater proportionate increase in the excretion of inorganic sulphates.

While the excretion of ammonia fluctuated, that of amino-acids and uric acid generally rose. Ethereal sulphate tended to diminish slightly. Creatinine and neutral sulphur remained practically constant, any increase being in no way proportional to the total increase of nitrogen or sulphur respectively.

3. Definite traces of creatine were observed particularly during phases of increased catabolism.

4. Traces of heat-coagulable protein were occasionally found, particularly during the time of the maximum excretion of the nitrogenous catabolites.

In conclusion, I wish to thank Prof. E. P. Cathcart for his helpful criticism. My thanks are also due to Prof. P. Patterson and to Messrs Patrick, Taylor and Stevenson for supplying me with suitable patients. Further, I wish to thank my nursing staff for their active co-operation in these and other previous experiments.

REFERENCES.

- Benedict (1907). *Carnegie Inst. Washington*, Pub. 77.
- and Osterberg (1923). *J. Biol. Chem.* **56**, 229.
- Bürger (1919). *Z. ges. exp. Med.* **9**, 361.
- Burian and Schur (1901). *Pflüger's Arch.* **87**, 239.
- Cameron and Gibson (1922). *Can. Med. Assoc. J.* **12**, 393.
- Campbell and Webster (1921). *Biochem. J.* **15**, 660.
- (1922). *Biochem. J.* **16**, 106.
- Cathcart and Burnett (1926). *Proc. Roy. Soc. Lond. B* **99**, 405.
- Kennaway and Leathes (1908). *Quart. J. Med.* **1**, 416.
- Cuthbertson (1929). *Biochem. J.* **23**, 1328.
- (1930). *Biochem. J.* **24**, 1244.
- Dunlop, Paton, Stockman and Macadam (1897). *J. Physiol.* **22**, 68.
- Folin (1905). *Amer. J. Physiol.* **13**, 45, 66 and 117.
- Greene (1919). *J. Biol. Chem.* **39**, 435.
- Gregor (1900). *Z. physiol. Chem.* **31**, 98.
- Hartmann (1924). *Arch. ges. Physiol.* **204**, 613.
- Hirst and Imrie (1928). *Quart. J. Med.* **22**, 153.
- Kocher (1914). *Deutsch. Arch. klin. Med.* **115**, 82.

- Leathes (1906). *J. Physiol.* **35**, 205.
- Levine, Gordon and Derich (1924). *J. Amer. Med. Assoc.* **82**, 1778, also *Arch. Int. Med.* (1924), **33**, 425.
- Mandel (1928). Cited by Lusk (1928). *Science of nutrition*, 80.
- Morpurgo (1897). *Arch. Path. Anat.* **150**, 502.
- von Noorden (1907). *The pathology of metabolism*, 290. (Heinemann, London.)
- Paton (1905). *J. Physiol.* **33**, 1.
- (1910). *J. Physiol.* **39**, 485.
- Pohl (1917). *Biochem. Z.* **78**, 200.
- Rakestraw (1921). *J. Biol. Chem.* **47**, 565.
- Sandiford, Sandiford, Deuel and Boothby (1926). *J. Biol. Chem.* **67**, xxiv.
- Shaffer (1907). *J. Biol. Chem.* **3**, xiii.
- (1908, 1). *Amer. J. Physiol.* **22**, 445.
- (1908, 2). *Amer. J. Physiol.* **23**, 1.
- Sivén (1901). *Skand. Arch. Physiol.* **11**, 123.
- Spriggs (1907, 1). *Biochem. J.* **2**, 206.
- (1907, 2). *Quart. J. Med.* **1**, 63.

XXXI. SELECTIVE FERMENTATION. ALCOHOLIC FERMENTATION OF MIXTURES OF GLUCOSE AND FRUCTOSE BY BREWER'S AND SAUTERNE YEASTS.

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IN recent years there has been a revival of interest in the phenomena of selective fermentation of mixtures of glucose and fructose by brewer's and other yeasts. Contributions have appeared from Willstätter and Sobotka [1922], Fernbach, Schoen and Mori [1927], Ivecovich [1930], Hopkins [1928], and Sobotka and Reiner [1930, 2]. Convenient summaries of the known phenomena appear in each of the last two papers. Broadly speaking, the facts calling for explanation are as follows:

(1) that glucose and fructose in separate solutions are fermented at approximately equal rates by living yeasts;

(2) that in mixtures of the two sugars, glucose is fermented faster than fructose by brewer's and most yeasts, but the reverse is the case with certain Sauterne yeasts; the selectivity was expressed by Hopkins [1928] by the formula

$$K_{G/F} = \frac{\ln a_g - \ln (a_g - x_g)}{\ln a_f - \ln (a_f - x_f)}$$

where a_g and a_f are the initial concentrations of glucose and fructose respectively, and x_g and x_f the concentrations fermented in a given time;

(3) that fructose alone is specifically esterified at least as rapidly as glucose by yeast juice or zymase prepared from brewer's yeast, and more rapidly in the presence of suitable concentrations of inorganic phosphates.

From a consideration of facts (1) and (2), it must be concluded that the total rate of fermentation is controlled by one of the reactions succeeding the stage at which selection occurs. The later reactions are apparently the same for both hexoses. The fact of selective fermentation, indeed, strongly supports the idea that the first reaction of fermentation in the living yeast cell is more rapid than a subsequent one. This is in agreement with the results of Harden and Young's work with yeast juice. Since such a majority of yeasts prefer glucose, Sauterne yeasts should be regarded as the exception. Neglecting this for the present, and considering together facts (2) and (3), the anomaly seems to be presented by brewer's and most other yeasts, and the author's attention was firstly directed to this.

It was thought possible that the rate of diffusion of one sugar into the

cell may be influenced by reaction products of the other sugar or even that one sugar may be produced from the other within the cell. For example, Harden and Young [1910] found that fructose is produced from hexose-diphosphate by the action of an enzyme, hexosephosphatase, in yeast juice. If such fructose is produced within the living cell, it would be produced at a definite rate per cell. More than one explanation of the selective action of brewer's yeast could be advanced on this hypothesis. The production of one sugar from the other *via* hexosephosphate at a fixed rate per cell should be independent of the concentrations of glucose and fructose in the external medium over wide range, so that the value of K should vary if widely differing initial proportions of glucose and fructose are used. This, however, was found not to be the case (Exp. 1).

It was shown by Kuhn and Münch [1925] that saccharase is of two types, that of brewer's yeast for example which is inhibited by fructose, and that of *e.g.* *Aspergillus oryzae* which is inhibited by glucose. Weidenhagen [1930] terms these two types respectively β -fructosidase and α -glucosidase. The possibility arises that the saccharases of brewer's and Sauterne yeasts are respectively of these two types, and in some way function to inhibit the fermentation of the respective sugars with which they may be assumed to combine. Investigation gave the following results.

(1) The saccharases of both brewer's and Sauterne yeasts were inhibited about twice as much by fructose as by glucose (Exp. 2). This was referred to by the author in a previous paper [Hopkins, 1930].

(2) Brewer's yeast, treated to reduce its saccharase activity relatively to its fermentative power by the method of Willstätter, Lowry and Schneider [1925], exhibited much the same selective power as before (Exp. 3). Sobotka and Reiner [1930, 2] have recently reported a similar result.

(3) Zymin prepared from brewer's yeast ferments glucose faster than fructose in a mixture, and this preference was undiminished by the addition of saccharase in the form either of a crude yeast autolysate (too old to contain any co-zymase) or of purified saccharase (Exp. 4). It should be noted, however, that the selective preference for glucose exhibited by zymin may be due simply to the production of fructose by hexosephosphatase.

The rate of fermentation of glucose and fructose in separate solutions. The approximate equality of the rates of fermentation in separate solutions by living yeast has been proved by experiments in which the reactions were of the zero-molecular type, *viz.* the rates measured were functions of the concentration of yeast and of one of the later reactions of the fermentation. Unless the sugar concentration is low enough to introduce a new limiting factor, no difference between the rates for the two separate sugars is likely to be found. It was found, however, that:

(1) Brewer's yeast ferments glucose faster than fructose when the concentration of sugar in the fermenting mixture is well below 1 %, whereas approximate equality in rate was observed above 1 % concentration.

(2) Sauterne yeast ferments glucose more slowly than fructose at any concentration up to 10 % (the maximum tested), but more decidedly so as the concentration falls (Exp. 5).

Influence of temperature on the value of $K_{G/F}$. It was observed by Hopkins [1928] that the selectivity of brewer's yeast was greater as temperature was lower. This has been confirmed, and values of $K_{G/F}$, 2.59 at 8°; 2.48 at 20°; 2.30 at 31° were obtained. Sauterne yeast yielded corresponding values for $K_{G/F}$, 0.051, 0.071, 0.098 at these temperatures respectively. The values of K were compared as far as possible at corresponding stages of the fermentations (Exp. 6). These results led to the hypothesis that brewer's yeast exerts a selective preference for that constituent of fructose which increases in proportion to rise in temperature. It is well known that the specific rotation of fructose becomes less negative by 0.64° per degree rise in temperature. This is explained by a change in the relative proportions of the constituent forms of fructose.

The mutarotation of fructose and glucose when partly fermented by the yeasts. Fructose, in accordance with the above hypothesis, mutarotated in the positive direction when brewer's yeast was used, and in the negative direction when Sauterne yeast was used. Glucose when partially fermented by brewer's yeast, showed a mutarotation in the positive direction, the value of $[\alpha]_D^{20}$ being + 49°. Willstätter and Sobotka [1922] obtained a similar result with a Munich brewery yeast. Again, Sauterne yeast acted in the converse manner, the glucose possessing $[\alpha]_D^{20} + 54^\circ$ at interruption.

In any explanation of the general phenomena of selective fermentation these results must be taken into account. The simplest inference to be drawn from the mutarotations of glucose is that brewer's yeast selectively prefers the α -form ($[\alpha]_D + 110^\circ$) and Sauterne yeast the β -form ($[\alpha]_D + 19^\circ$) of glucose. On this assumption the values of $K_{\alpha/\beta}$ for the brewer's yeast are 1.4 to 1.5 at the stage of 30 to 40 % of total glucose fermented. Willstätter and Sobotka's result for the corresponding stage of fermentation was only slightly higher. The selective action of the yeasts on fructose constituents is more significant. It may be that only α - and β -forms of normal fructose exist in an aqueous solution. β -Fructose has a specific rotation of -130° [Nelson and Beegle, 1919], the other form has not been isolated, but is presumably present only in small proportion at low temperatures, but increasingly with rise of temperature. However, the striking change in optical rotation of fructose with change of temperature, a phenomenon not paralleled in glucose or the other familiar sugars, strongly suggests that the unidentified form is one the formation of which from normal fructose involves a not inconsiderable energy change. Brown and Pickering [1897] found that 1 g. of glucose (α) evolves from 0.7 to 0.9 calorie in mutarotating to equilibrium, whereas 1 g. of fructose absorbs from 4.6 to 5.4 calories (probably only about 0.1 g. of fructose actually changes to the γ -form). Normal fructose, according to the now generally accepted formula of Haworth consists of an amylene oxide or δ -ring (pyranose).

The other form may be a butylene oxide or γ -ring (furanose). The latter probably possesses a + rotation as would be expected from considerations of the specific rotations of the tetramethyl δ - and γ -fructoses. The application of Hudson's lactone rule to γ -fructose would also indicate a + rotation. If this is so this form of fructose, if present, would be present in very low proportion in aqueous solutions of fructose at ordinary temperatures. (It should be noted that Morgan and Robison [1928] found that the hexose of hexosediphosphate is γ -fructose.)

The hypothesis is now advanced that brewer's and presumably most yeasts are specific for the normal aldo-hexoses, glucose and mannose, and for this unidentified, but probably γ -form of fructose. If, as seems probable, this latter is present only in relatively small proportion in fructose solutions, it would necessarily be taken up slowly in a selective fermentation of a mixture of glucose and fructose. Conversely, Sauterne yeasts are specific for normal fructose (or β -fructose on the simple theory of only α - and β -forms) and ferment glucose specifically more slowly than normal fructose. If this hypothesis should prove correct, the problem of the selective fermentation of these sugars by various yeasts resolves itself into a more normal one, viz. that brewer's and most yeasts are specific for certain hexoses, those derived directly from maltose, sucrose, etc., and Sauterne yeasts for normal fructose as it occurs in grapes.

EXPERIMENTAL.

The glucose and fructose used in these experiments were found to give $[\alpha]_D^{20} + 52.6^\circ$ and -93.0° respectively. The brewer's yeasts were all top fermentation yeasts obtained from Scottish breweries. Exps. 5 to 8 (Tables IV, V, VI and I, Nos. 11-20) were performed within a few weeks of each other with yeast obtained from the same source. The Sauterne yeast used in the same series was isolated from a bottle of Sauterne wine (1928), from which the yeast used in Exp. 2 had been previously isolated in 1928. It was grown in a medium of grape must and fructose.

Exp. 1. Selective fermentations by brewer's yeast with varying initial proportions of glucose and fructose.

Fermentations with yeasts A and B were performed at 25° , and samples withdrawn at intervals, centrifuged and filtered. The polarimetric readings were taken at 20° after allowing time for the rotations to stabilise at that temperature, and the reducing powers by the method of Lane and Eynon. The results are given in Table I (1-7).

Exp. 2. Inhibition of the saccharase of brewer's and Sauterne yeasts by glucose and fructose respectively.

The inversions were performed at 20° and all polarimetric readings were made at the same temperature which was never allowed to change.

Table I. *Selective fermentation of glucose and fructose by various types of yeast and under various conditions.*

Exp. 1.	1	Yeast and temp.	Time (hours)	% fer- mented of total sugars	Sugars unfermented		$K_{G/F}$
					Glucose	Fructose	
Exp. 1.	1	Brewer's A 25°	—	0	10.06	2.45	—
			—	39	5.79	1.88	2.08
			—	63	3.25	1.41	2.03
	2	"	—	0	6.30	5.98	—
			—	54	1.99	3.56	2.22
			—	71	0.96	2.61	2.26
	3	"	—	0	2.64	10.00	—
			—	77	0.24	2.66	1.79
	4	Brewer's B 25°	—	0	9.98	1.11	—
			—	80	1.725	0.465	2.02
	5	"	—	0	9.00	1.84	—
			—	77	1.624	0.816	2.10
	6	"	—	0	5.04	5.16	—
			—	23	3.56	4.36	2.07
			—	77	0.833	2.22	2.13
	7	"	—	0	1.50	8.40	—
			—	31	0.721	6.15	2.30
Exp. 3.	8	Brewer's C Normal	0	0	4.93	4.88	—
			2½	47	1.85	3.32	2.54
			5½	79	0.40	1.64	2.32
	9	Same (acid treatment)	0	0	4.95	4.90	—
			3	26	3.24	4.10	2.38
			5½	45	2.06	3.34	2.29
	10	Same (alkali treatment)	0	0	4.93	4.88	—
			2½	33	2.70	3.84	2.51
			5½	56	1.42	2.88	2.36
Exp. 6.	11	Brewer's D 9°	0	0	4.23	4.17	—
			7	25	2.61	3.67	3.80
			22	70	0.61	1.91	2.48
	12	20°	0	0	4.79	4.72	—
			4	37	2.40	3.57	2.48
	13	31°	0	0	4.94	4.86	—
			4	48	1.90	3.23	2.33
			6	70	0.77	2.17	2.30
	14	8°	0	0	4.13	4.07	—
			23	31	2.34	3.29	2.67
			52	71	0.536	1.85	2.59
	15	34°	0	0	4.13	4.07	—
			4	38	2.04	3.02	2.36
			7½	71	0.64	1.76	2.22
	16	Sauterne (isolated 26. x. 29) 8°	0	0	4.09	3.98	—
			32	43	3.90	0.72	—
	17	34°	0	0	4.09	3.98	—
			5	50	3.73	0.27	0.034
	18	Sauterne 7°	0	0	9.26	2.06	—
			8	13	8.82	1.07	0.074
			19	25	8.26	0.22	0.051
	19	20°	0	0	9.99	2.21	—
			8	22	8.99	0.50	0.071
	20	31°	0	0	10.35	2.28	—
			8	12	9.79	1.29	0.098

1. 100 cc. of 0.146 *M* sucrose + 2.0 g. of pressed yeast + 4 cc. chloroform.
2. 100 cc. of 0.146 *M* sucrose } + 4.0 g. of pressed yeast + 8 cc. chloroform.
+ 100 cc. of 0.146 *M* glucose }
3. 100 cc. of 0.146 *M* sucrose } + 4.0 g. of pressed yeast + 8 cc. chloroform.
+ 100 cc. of 0.146 *M* fructose }

25 cc. samples were withdrawn and the reaction stopped by addition to 2 cc. of *N* NaOH and 1 cc. of alumina cream.

Table II. *Inhibition of inversion of sucrose by yeasts in the presence of glucose and fructose.*

		Polarimetric readings (2 dm. tube) at 20°					
		Sucrose + glucose			Sucrose + fructose		
		(0.146 <i>M</i>)	(0.146 <i>M</i>)		(0.146 <i>M</i>)	(0.146 <i>M</i>)	
		Sucrose (0.146 <i>M</i>)	Inhibition		Inhibition		
			%		%		
Brewer's yeast	Time (min.)						
	0	+ 6.09°	+ 5.57°	—	- 1.41°	—	—
	60	+ 4.09°	+ 4.08°	25.5	- 2.23°	59	
	104	+ 3.10°	+ 3.37°	26.3	- 2.73°	56	
Sauterne yeast	149	+ 2.10°	+ 2.68°	27.5	- 3.35°	51	
	0	+ 5.88°	+ 5.38°	—	- 1.36°	—	—
	34	+ 3.62°	+ 3.75°	28	- 1.99°	72	
	60	+ 2.02°	+ 2.63°	29	- 2.92°	60	

Exp. 3. Selective fermentation by yeast weak in saccharase.

Washed pressed brewer's yeast was treated with 0.2 *N* sulphuric acid for one hour and its saccharase value substantially reduced. Another portion suffered similarly by treatment with 0.05 *N* sodium hydroxide. A third portion was steeped in water. Selective fermentations were performed with the three yeasts immediately after washing. The results appear in Table I, Exps. 8, 9, 10.

Exp. 4. Influence of saccharase on selective fermentation by zymin, prepared from brewer's yeast.

To 200 cc. of a solution of the mixed sugars were added 10 g. of zymin; after mixing the solution was divided into two equal portions and a saccharase preparation added to one, and the same volume of boiled preparation to the other. A drop of toluene was added to each, and fermentation allowed to proceed at 25°. After a suitable gas evolution had occurred, fermentation was arrested by clearing with alumina cream, boiling and clearing afresh. The residual solution was analysed as for the yeast fermentations, and finally fermented to completion with living yeasts and again analysed, the latter results being deducted from the previous ones. In *Exp. A*, zymin from lager brewery yeast was used, and the saccharase preparation was a crude autolysate of yeast, too old to contain any co-zymase. In *Exp. B*, top brewery yeast zymin and a purified saccharase obtained from yeast autolysate by adsorption with aluminium hydroxide (Willstätter and Kraut) and eluted with dilute ammonia.

Table III. *Selective fermentation by zymín with and without added saccharase.*

	Saccharase preparation		Sugars g. per 100 cc.	
			Glucose	Fructose
A. Lager yeast zymín	Crude yeast	Initial conc.	3.80	3.80
	autolysate	Fermented by zymín	0.70	0.35
	Control	Initial conc.	3.80	3.80
		Fermented by zymín	0.62	0.28
B. Top yeast zymín	Purified	Initial conc.	3.33	3.02
	saccharase	Fermented by zymín	1.97	0.18
	Control	Initial conc.	3.33	3.02
		Fermented by zymín	2.55	0.30

Exp. 5. Velocity of fermentation of glucose and fructose in separate solutions.

This was measured at 30° in the apparatus devised by Slator [1906]. The highest rate observed in the experiments with both brewer's and Sauterne yeasts was with fructose at 3.5 % in the fermenting mixture, and this was adopted as a standard in each case. The results, expressed in terms of these standards as 100 are given in Table IV. Autolysis rates have been deducted.

Table IV. *Relative rates of fermentation of glucose and fructose at 30°.*

	Sugar g. per 100 cc. of fermenting liquid	Glucose	Fructose
Brewer's yeast	0.20	51	31
	0.40	76	52
	1.00	89	90
	3.50	95	100
Sauterne yeast	0.20	—	43
	0.40	10	75
	0.50	15	79
	1.00	34	88
	3.50	54	100
	9.00	65	95
	10.50	71	87

Exp. 6. Selective fermentations of glucose and fructose at different temperatures.

In this experiment it is desirable to avoid either excessively fast or slow fermentations. In the former case the fermentation may be fast enough to compare with the rate of mutarotation of glucose, and in the latter, prolonged exposure to different temperatures may cause variations in the character of the yeast. Samples were withdrawn from the fermentations at intervals by rapid centrifugation followed by filtration. The procedure was as in Exp. 1, except that the reducing sugars were estimated by the Hagedorn-Jensen method as modified by Hanes, using the table of values furnished by Sobotka and Reiner [1930, 1]. In the case of fermentations with Sauterne yeast it was found desirable to start with a much higher proportion of glucose than of fructose, otherwise experimental errors are unduly large, and the appropriate juncture at which to withdraw a sample is difficult to judge. The results are

given in Table I, Exps. 11 to 20. The concentrations used in Nos. 18–20 were chosen with due regard to the results of Exp. 5.

Exp. 7. Mutarotation of glucose solution when partly fermented by yeast.

The experiments with glucose were performed by much the same procedure as was adopted by Willstätter and Sobotka [1922] except that the percentage of sugar fermented was determined by a separate withdrawal, and the addition of chloroform to arrest fermentation was omitted. About 200 g. of well-washed pressed yeast were added to an equal weight of 15 % glucose solution, and fermentation was allowed to proceed at 30° with frequent stirring. After a suitable time a few cc. of *N* acetic acid and a quantity of powdered ice were added and the liquid filtered rapidly through a large filter previously layered with alumina cream (free from salts, and neutral). The first runnings were rejected. The rotation could be read within two or three minutes of zero time, the latter being taken as the mean of the time of commencement and completion of filtration of the solution required to fill the observation tube. Fermentation was still proceeding in the funnel and was only arrested as the liquid passed through the filter. The acetic acid was added to ensure the attainment of a p_H of about 3.6 at which the rate of mutarotation is at a minimum. The fermentations with Sauterne yeast were performed at 20° and no ice added. It was found that less error was involved in this way, since when glucose is suddenly chilled a slow fall in rotation follows. The values of the constant $K_{\alpha/\beta}$ were calculated as by Willstätter and Sobotka, assuming $[\alpha]_D$ for α -glucose + 110°, for β -glucose + 19° and for the equilibrium solution + 52.6°.

Table V. *Mutarotation of glucose solutions partly fermented by yeast.*

% sugar fermented	Initial (extra- polated from t_1, t_2, \dots)	Polarimeter readings					$[\alpha]_D$ at time of arresting fermen- tation	$K_{\alpha/\beta}$
		t_1	t_2	t_3	t_4	Final t_∞		
Brewer's yeast:		6 min.	12 min.	60 min.				
28.7	9.67°	9.73°	9.77°	9.88°	—	10.20°	+ 49.8°	1.53
		3 min.	5 min.	60 min.				
31.9	9.12°	9.24°	9.28°	9.46°	—	9.74°	+ 49.2°	1.47
		3 min.	14 min.	27 min.	100 min.			
38.6	8.20°	8.28°	8.38°	8.44°	8.58°	8.78°	+ 49.1°	1.38
		3 min.	14 min.		107 min.			
41.3	7.80°	7.90°	8.05°	—	8.19°	8.40°	+ 48.8°	1.38
Sauterne yeast:		4 min.	45 min.	100 min.	145 min.			
—	12.31°	12.29°	12.19°	12.14°	12.09°	11.99°	+ 54.0°	—
		3 min.	45 min.	100 min.				
—	11.60°	11.58°	11.52°	11.50°	—	11.25°	+ 54.2°	—
		4 min.	48 min.					
—	5.75°	5.73°	5.68°	—	—	5.56°	+ 54.4°	—

Exp. 8. Mutarotation of fructose solution when partly fermented by yeasts.

The procedure was as for glucose, with the following differences. In working with fructose much greater speed of observation is necessary than with glucose owing to the greater rate of mutarotation. It is also necessary to keep the temperature absolutely constant throughout fermentation, filtration and polarimetric observations. The experiments were performed at laboratory temperature, about 10°. It was not possible to calculate the values of K since the proportions of the constituents of fructose solution at equilibrium are unknown. Should the latter information become available, the data here presented may be of possible use in this connection.

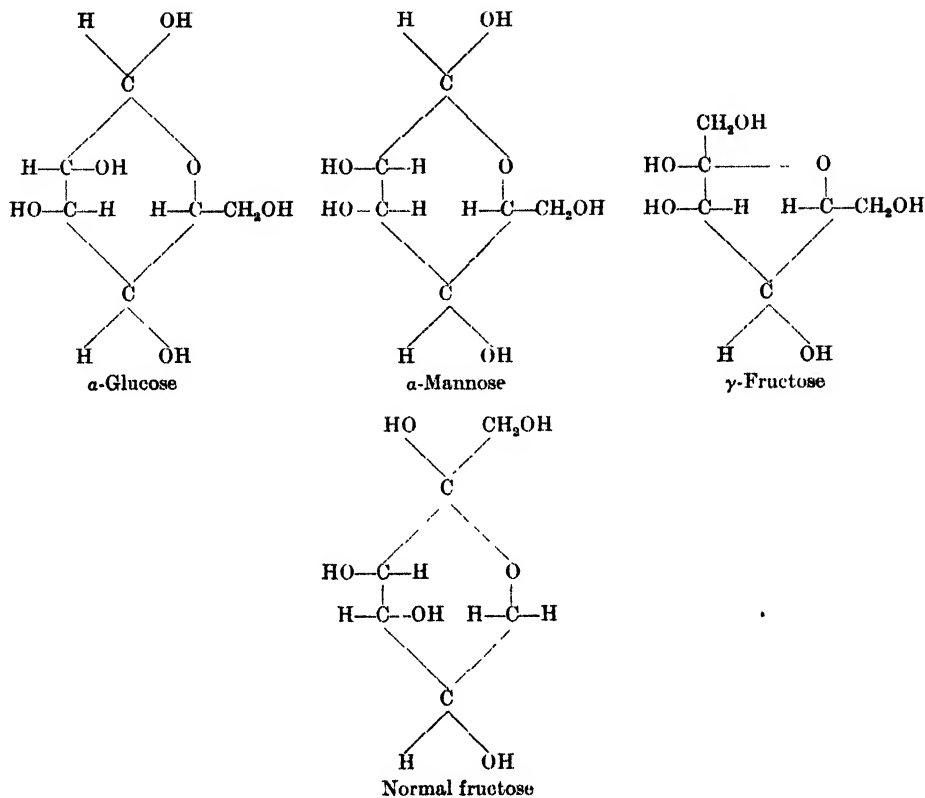
Table VI. *Mutarotation of fructose solutions partly fermented by yeast.*

% sugar fermented	Initial (extra- polated from t_1, t_2, \dots)	Polarimeter readings					$[\alpha]_D^{20}$ at time of arresting fermen- tation
		t_1	t_2	t_3	t_4	Final t_∞	
Brewer's yeast:							
13	9.37°	2 min. 9.30°	4 min. 9.27°	17 min. 9.10°	38 min. 8.98°	8.98°	- 97.1°
21	8.55°	2 min. 8.46°	5 min. 8.38°	21 min. 8.20°	40 min. 8.14°	8.14°	- 97.4°
25	8.12	2 min. 8.05°	5 min. 7.99°	15 min. 7.86°	36 min. 7.79°	7.78°	- 97.1°
54	4.90°	2 min. 4.81°	4 min. 4.76°	23 min. 4.57°	50 min. 4.52°	4.51°	- 100.8°
56	5.06°	2 min. 4.94°	5 min. 4.84°	20 min. 4.66°	90 min. 4.64°	4.62°	- 101.8°
Sauterne yeast:							
44	6.00°	4 min. 6.05°	8 min. 6.08°	24 min. 6.12°	—	6.14°	- 90.8°
50	13.20°	3 min. 13.26°	—	42 min. 13.54°	—	13.60°	- 90.3°
50	8.56°	2 min. 8.64°	5 min. 8.73°	25 min. 8.93°	—	8.96°	- 89.0°
57	7.10°	2 min. 7.16°	3 min. 7.19°	28 min. 7.62°	75 min. 7.65°	7.72°	- 85.6°

NOTE ON THE CONFIGURATIONS OF GLUCOSE, MANNOSE AND FRUCTOSE.

In view of the foregoing hypothesis, it is interesting to compare the configurational formulae of the three hexoses for which yeasts of the brewer's yeast type are supposed to be specific. It will be seen that, apart from the reducing carbon atom and its attachments, the three hexoses, glucose, mannose and γ -fructose are identical in configuration. The suggestion put forward by E. F. Armstrong that the three fermentable hexoses owe this property to certain features of their configurations which they have in common, could be modified to fit the hypothesis now put forward. Normal fructose has little

in common with the other three hexoses. The formulae of Haworth have been adopted.



SUMMARY.

1. The factor $K_{G/F}$ for brewer's yeast was not appreciably influenced by the relative proportions of glucose and fructose in the original solution.
2. The factor was not influenced by modification of the saccharase activity of brewer's yeast by the method of Willstätter, Lowry and Schneider.
3. The saccharase of Sauterne yeast resembled that of brewer's yeast in that it was inhibited more strongly by fructose than by glucose.
4. The addition of saccharase to a mixture of glucose and fructose undergoing fermentation by zymine (brewer's) did not affect the selectivity.
5. The factor $K_{G/F}$ for brewer's yeast decreased with rise in temperature of fermentation, whilst that for Sauterne yeast increased.
6. Brewer's yeast ferments glucose faster than fructose in separate solutions when the concentration of sugar is less than 1 %. Sauterne yeast ferments fructose faster than glucose in separate solutions at all concentrations up to 10 %, but especially at low concentrations.
7. The mutarotation of partly fermented solutions of the sugars is for

glucose by brewer's yeast in the positive direction, glucose by Sauterne yeast in the negative, fructose by brewer's yeast in the positive, and fructose by Sauterne yeast in the negative direction.

8. The hypothesis is advanced that brewer's yeast and most yeasts are specific for that form of fructose which is present in small proportion, but which increases with temperature, possibly a γ -form, whereas Sauterne yeasts are specific for the normal form.

In conclusion I wish to express my thanks to Dr W. O. Kermack for the interest he has shown in this work, and for useful criticisms of the theories.

REFERENCES.

- Brown and Pickering (1897). *J. Chem. Soc.* **71**, 768.
Fernbach, Schoen and Mori (1927). *Compt. Rend. Acad. Sci.* **184**, 168, 551.
Harden and Young (1910). *Proc. Roy. Soc. Lond. B* **82**, 321.
Hopkins (1928). *Biochem. J.* **22**, 1145.
—— (1930). *J. Inst. Brew.* **36**, 193.
Ivecovich (1930). *Biochem. J.* **24**, 4.
Kuhn and Munch (1925). *Z. physiol. Chem.* **150**, 220.
Morgan and Robison (1928). *Biochem. J.* **22**, 1270.
Nelson and Beegle (1919). *J. Amer. Chem. Soc.* **41**, 559.
Slator (1906). *J. Chem. Soc.* **89**, 128.
Sobotka and Reiner (1930, 1). *Biochem. J.* **24**, 394.
—— (1930, 2). *Biochem. J.* **24**, 926.
Weidenhagen (1930). *Fermentforsch.* **11**, 155.
Willstatter, Lowry and Schneider (1925). *Z. physiol. Chem.* **146**, 158; **150**, 287.
—— and Sobotka (1922). *Z. physiol. Chem.* **123**, 170, 164.

XXXII. THE PROTEOLYTIC ENZYMES OF GREEN MALT.

II. ACTION OF THE PROTEINASE ON EGG-ALBUMIN, CASEINOGEN, EDESTIN AND FIBRIN AT DIFFERENT REACTIONS.

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DEFINITE progress in the direction of clarifying our ideas on the nature of the proteolytic enzymes of green malt has been made in the past two years, during which no less than six papers appeared [Mill and Linderstrøm-Lang, 1929; Lüers and Malsch, 1929; Hopkins, 1929; Linderstrøm-Lang and Sato, 1929, 1, 2; Hopkins and Burns, 1930]. In their main conclusions these authors agreed that green malt contains (1) a protease or proteinase (to adopt the nomenclature of Grassmann) which appears to attack edestin, gelatin, crystalline egg-albumin and Witte peptone, and (2) at least one peptidase which attacks the dipeptide leucylglycine. According to Linderstrøm-Lang and Sato [1929, 2] there is a second peptidase present, which attacks alanyl-glycine and, unlike the previous one, is not inhibited by phosphates. The present communication is concerned with the activities of the proteinase.

It seems desirable, in investigations of the relations between enzyme activity and p_H , to use as purified a preparation of the enzyme as possible. Certain difficulties however present themselves. The yield of proteinase in the preparations obtained by the adsorption and elution methods so far tried is rather low, and the quantity of mineral salts present in the elutions is sufficient to influence the properties of the substrates. For instance, it was shown by Michaelis and Szent-Györgyi [1920] that the ions of certain salts influence the p_H of maximum precipitation of caseinogen. This is in agreement with certain observations of Hardy in connection with globulins. The present authors have also observed that phosphates and citrates, the former of which are used for elution of the enzyme, and both of which are used as buffers to stabilise p_H , inhibit the activity of the proteinase at p_H 4.7, but whether this is due to a cause such as that observed by Michaelis and Szent-Györgyi or to some other inhibitive effect was not investigated. For the present, the activity of the proteinase has been investigated using ordinary cold aqueous extract of green malt as the active agent, and in the absence of added buffers except in Exp. 1. The main object of the work was to ascertain

if the proteinase attacks its protein substrates optimally at their isoelectric points, *i.e.* is specific for the zwitterions, as suggested by Willstätter, Grassmann and Ambros [1926], in the cases of papain and bromelin.

In allowing the enzyme to act at 40° or thereabouts, it is necessary to adopt a short time of action, since the enzyme, in the presence of malt extract alone, is susceptible to fairly rapid inactivation or destruction when maintained at this temperature at a reaction outside the range of p_H 3.8 to 6.0. This was investigated by Hopkins and Burns [1930], who preserved an aqueous extract of green malt with toluene at various reactions at 40°. It was found that the proteinase is destroyed in the presence of free alkali (hydroxyl ions) and of excessive acidity, its optimum stability being at p_H 4.4 to 5.0, or near the isoelectric point (presumably) of the malt extract proteins. The activity was reduced to one-half in 24 hours at p_H 6.0, and in 48 hours at p_H 3.8. In order to avoid any displacement of the p_H of the optimum activity, and any serious distortion of the p_H -activity curves, the experiments recorded here were performed by allowing the enzyme to act on the substrates over a range of p_H for 8 hours. Except in the case of edestin, the proteolysis was measured by the amount of protein rendered soluble or non-coagulable, as the case may be, at its isoelectric point. This eliminated the measurement of any action due to the peptidases. The results may be briefly stated here.

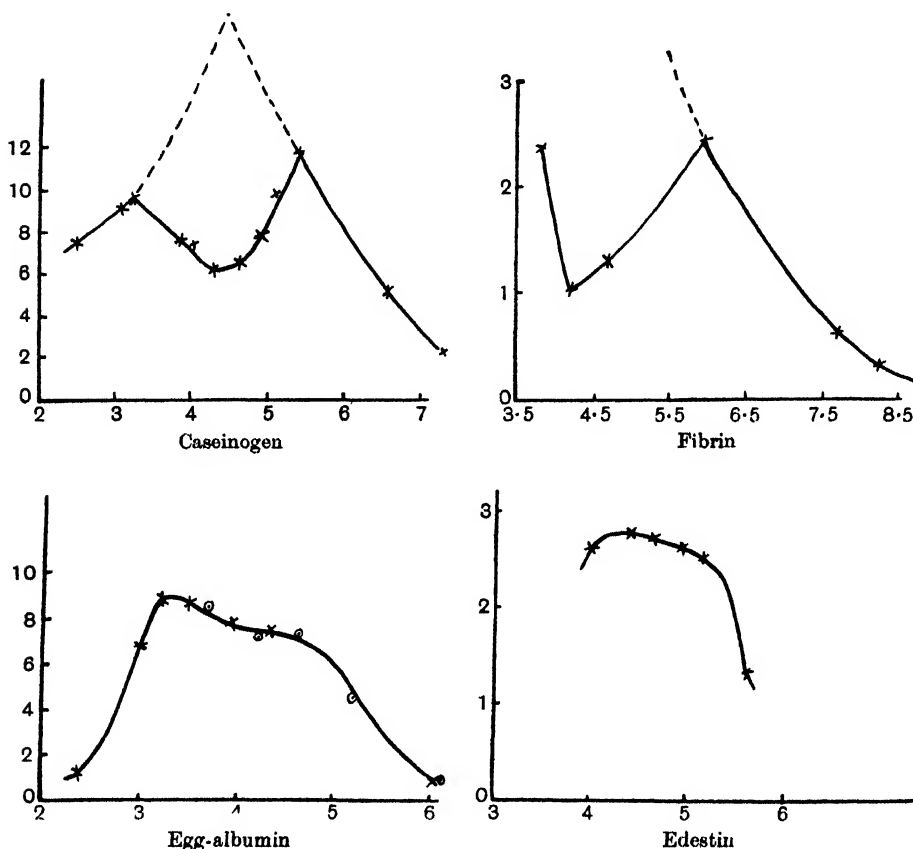
Egg-albumin. It was found that, at p_H 4.6 and 46°, proteolysis was proportional to time up to 6 hours or more (Exp. 1). The optimum activity was exhibited at p_H 3.3 in the presence of phosphate-citrate buffers (Exp. 2) and at a point more acid than p_H 3.8 in the absence of added buffers (Exp. 3). The isoelectric point of egg-albumin is at p_H 4.8 according to Sørensen. The activity at p_H 3.87 in the absence of added buffers was 1.23 times that at p_H 4.8 (Fig. 1).

Caseinogen. At 46° and p_H 5.1 proteolysis was proportional to time up to 9 hours (Exp. 5). Two optima of activity were observed at p_H 3.35 and 5.57, *i.e.* on each side of the isoelectric point, which is 4.62 according to Michaelis and Pechstein [1912] (Exps. 6 and 7).

Edestin. The relation between proteolysis and p_H for edestin was investigated by Mill and Linderström-Lang [1929] who found the optimum to be at p_H 4.3. This was obtained in the presence of chlorine ions, the protein being in solution in hydrochloric acid or sodium chloride. These results have been confirmed using a shorter period of time for the reaction (Exp. 9). In view of the work of Michaelis and Szent-Györgyi [1920] already referred to, the experiment was repeated using acetic acid instead of hydrochloric acid (Exp. 10). The point of maximum precipitation of edestin is unaffected by sodium acetate, whereas in the presence of sodium chloride it is shifted in the acid direction. It was found, however, that proteolysis increased with acidity up to the maximum attainable with acetic acid as the sole acid present.

Fibrin. At 46° and p_H 4.8 proteolysis was proportional to time for 2 hours only (Exp. 12). Two optima of activity were observed at p_H 3.8 and 6.0, *i.e.*

on each side of the zone of maximum precipitation of the protein under the conditions obtaining. The isoelectric point of fibrin is generally stated to be about 6.8, for which reason fibrin was used. It was very difficult to dissolve, and, after solution had been effected under somewhat drastic conditions, the zone of maximum precipitation was at about p_H 4.8.



Ordinates. Proteolysis as cc. $N/10$ HCl.

Abcissae. p_H .

The broken lines represent the forms that the curves would presumably take, if the substrates did not flocculate within the zones of minimum solubility.

Readings from Table II are represented by crosses on the egg-albumin curve, readings from Table III (after reduction to appropriate values of the ordinate) are represented by encircled dots.

The diagram illustrates the results, ordinates representing proteolysis, the abscissae p_H .

DISCUSSION OF THE RESULTS.

The p_H optima of proteinase activity under the conditions adopted do not coincide with the isoelectric points of the respective substrates. In the cases of caseinogen and fibrin, the protein being almost insoluble over a zone of p_H including the isoelectric point, it is only to be expected that proteolysis

would be less vigorous here, since the substrate is partly or wholly in suspension, and the concentration in solution is low enough to limit the rate of enzyme action. This cannot be avoided, since, if the very small fraction of protein which remains in solution at the point of maximum precipitation were alone used as substrate, and proteolysis measured by the Willstätter or formaldehyde titration (which would be necessary in this case), we should probably be using neither caseinogen nor fibrin as substrate, but a small fraction of abnormal and possibly contaminated material. The p_H activity curves of both caseinogen and fibrin strongly suggest that, but for the insolubility of the substrate within this zone, the proteolytic action would have increased to an optimum within the zone, and in the case of caseinogen this would have approximated to the isoelectric point. However, egg-albumin, which is soluble at all reactions investigated, gives results indicating that the optimum is not at the isoelectric point (4.8) but at p_H 3.3 to 3.6. Edestin, although held in solution fairly completely by sodium chloride or acetate at all reactions investigated, showed greater susceptibility to proteolysis as acidity increased from p_H 5.5, whereas the isoelectric point of edestin is at p_H 5.5 to 6.0.

These varying results for the four substrates suggest much speculation on such lines as the state of molecular aggregation and osmotic pressure of the respective proteins at their isoelectric points and elsewhere, and the presence or otherwise of zwitterions or of un-ionised molecules in the various cases.

However, one observation is sufficiently striking to merit notice. It will be seen from the electrometric titration data given in Tables IV, VIII, XI and XIV that the p_H optimum for proteolysis of each protein approximates to that of maximum buffering power. The latter is given in the Tables, expressed as the Van Slyke coefficient $\Delta A/\Delta p_H$ or $\Delta B/\Delta p_H$, where ΔA and ΔB are increments of added acid and base, and Δp_H the corresponding change in p_H . The p_H range, corresponding to the maximum values of this coefficient for each protein, together with the results derived from the electrometric titrations of other observers, are summarised in Table XV. The only appreciable discrepancy is in the case of edestin, but even here the p_H optimum of proteolysis found is much nearer to that of buffering than to the isoelectric point. It seems that, when the p_H of maximum buffering power of the protein substrate falls within the range of activity of the malt proteinase, the optimum proteolysis will occur at or near that p_H . In the case of the proteins possessing an insoluble zone, the optimum is necessarily outside this insoluble zone. The proteinase appears to act on ionised protein, on both sides of the isoelectric point, and it is quite possibly specific for zwitterions where these exist. Further speculation is at present premature.

EXPERIMENTAL.

Preparation of the malt extract. Air-dried green malt was finely ground, extracted with 3 times its weight of water for 12 hours at laboratory tem-

perature and filtered. The extract used in the experiments, in which the formaldehyde titration was employed to measure proteolysis, was prepared from kiln-dried malt, which is unlikely to contain any quantity of the peptidases.

The substrates were crystalline egg-albumin (Merck), edestin from hemp-seed (Merck), caseinogen (a commercial product purified by Hammarsten's process), and fibrin prepared directly from blood and preserved in alcohol.

The buffer solution used in Exp. 2 was McIlvaine's phosphate-citrate, as described by Clark [1928].

All hydrogen ion measurements were performed by means of the quinhydrone electrode.

Exp. 1. Egg-albumin. Kinetics. 100 cc. of 6 % albumin + 20 cc. of acetate buffers (sodium acetate and acetic acid, $N/2$ with respect to each), p_H 4.6 + 50 cc. of malt extract, mixed and incubated at 46° . Control, the same with boiled malt extract. 25 cc. withdrawn at intervals and added slowly to 60 cc. of boiling water and boiled for 15 minutes, filtered, washed with boiling water, and total nitrogen determined in filtrate by the Kjeldahl process.

Table I.

Time (hours)	3	6	12	24	36
Action, cc. $N/10$ HCl (control deducted)	3.5	7.2	9.8	10.5	10.9

Exp. 2. Egg-albumin. Proteolysis at different reactions. 15 cc. of albumin + 15 cc. of malt extract + 10 cc. of phosphate-citrate buffer solution of appropriate p_H + $N/10$ HCl or NaOH as required + water to a total volume of 60 cc. After 8 hours at 46° , 30 cc. were withdrawn and coagulated as in Exp. 1, after addition of the required volume of $N/10$ NaOH or HCl. The latter was firstly calculated, and then a *pro rata* portion added to 20 cc. of the remaining solution and the resulting p_H tested to confirm that it was 4.8. The remaining portion of the reaction mixture was used for the determination of its p_H .

Table II.

p_H of reaction mixture	2.25	2.97	3.34	3.69	3.74	4.11	4.40	6.13	6.69	7.14	7.52	7.77
p_H of control	2.25	2.97	3.30	3.69	—	—	—	6.13	6.70	7.14	7.50	7.73
Proteolysis, cc. $N/10$ HCl (controls deducted)	1.0	5.5	8.6	8.0	7.9	7.0	6.4	1.2	0.8	0.6	0.5	0.3

Exp. 3. Egg-albumin. Proteolysis in absence of added buffers. This experiment was performed exactly as the previous one except that the phosphate-citrate buffers were omitted. All withdrawals were treated so as to contain the same concentration of NaCl at the time of coagulation.

Table III.

p_H of reaction mixture	3.87	4.32	4.73	5.28	6.18
p_H of control	3.73	4.30	4.73	5.28	6.19
Proteolysis, cc. $N/10$ HCl (controls deducted)	8.5	7.0	6.9	5.0	1.5

Exp. 4. Electrometric titration of the egg-albumin. Table IV gives the volumes of $N/10$ HCl or NaOH added to 1 g. of protein, and the p_H resulting. The buffer coefficients were calculated according to the formula of Van Slyke $\Delta A/\Delta p_H$ or $\Delta B/\Delta p_H$.

Table IV.

$N/10$ acid cc.	0.0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	5.0
p_H	4.89	4.55	4.30	4.06	3.87	3.67	3.49	3.31	3.09	2.64
$\Delta A/\Delta p_H$		1.47	2.00	2.08	2.63	2.50	2.78	2.78	2.27	2.22
$N/10$ base cc.	0.0	0.5	1.0	1.1	1.2	1.3	1.4			
p_H	4.89	5.31	6.03	6.27	6.55	6.83	7.09			
$\Delta B/\Delta p_H$		1.19	0.70	0.41	0.35	0.35	0.38			

Exp. 5. Caseinogen. Kinetics. 2 g. of caseinogen, suspended in 100 cc. of $N/20$ NaOH until dissolved, were brought to p_H 5.1 with $N/2$ HCl. 50 cc. of malt extract previously adjusted to p_H 5.1 were added, and the mixture incubated at 46° . At intervals 20 cc. were withdrawn, added to 20 cc. of acetate buffer solution (p_H 4.6), filtered, and total nitrogen determined on the filtrate. The control was treated similarly using boiled malt extract.

Table V.

Time (hours)	3.0	6.5	9.0
Action, cc. $N/10$ (control deducted)	4.2	7.8	10.7

Exp. 6. Caseinogen. Proteolysis at different reactions. 20 cc. of caseinogen (2 % in $N/20$ NaOH) + 15 cc. of malt extract + 19 cc. of $N/10$ HCl + respectively 0.0, 3.0, 5.0, 7.0, 8.0, 11.0 cc. of $N/10$ NaOH + water to 80 cc. Of this 20 cc. were withdrawn for p_H determination, and the remainder incubated at 46° for 8 hours. To each was now added the requisite volume of $N/10$ NaOH or HCl to bring to p_H 4.6 + water to the same total volume. Total nitrogen was determined on 50 cc. of the filtrate.

Table VI.

p_H of reaction mixture	2.58	2.97	3.35	3.82	4.10	5.34
Proteolysis, cc. $N/10$ HCl (controls deducted)	7.42	8.75	9.60	7.70	7.50	9.66

Exp. 7. Caseinogen. Proteolysis in presence of acetate. The procedure was exactly as in the previous experiment except that acetic acid was used instead of HCl.

Table VII.

p_H of reaction mixture	3.91	4.41	4.70	5.00	5.56	6.50	7.40
Proteolysis, cc. $N/10$ HCl (controls deducted)	7.6	6.2	6.6	7.9	11.7	5.16	2.2

Exp. 8. Caseinogen. Electrometric titration. Table VIII gives the volumes of $N/10$ HCl added to 0.2 g. of caseinogen in $N/10$ NaOH, and the resulting p_H . The buffer coefficients are also given.

Table VIII.

<i>N</i> /10 HCl cc.	8.0	8.4	8.6	8.8	9.0	9.2	9.4	9.6	9.8
p_H	8.37	6.89	6.47	6.31	5.93	5.49	5.15	4.71	4.16
$\Delta A/\Delta p_H$	0.27	0.48	1.25	0.53	0.45	0.58	0.45	0.36	
<i>N</i> /10 HCl cc.		10.0	10.5	11.0	11.5				
p_H		3.80	3.36	3.00	2.60				
$\Delta A/\Delta p_H$		0.55	1.14	1.40	1.25				

Exp. 9. Edestin. Proteolysis at different reactions. 1 g. of edestin was suspended in 50 cc. of *N*/10 HCl until dissolved. 10 cc. of the solution + 15 cc. of malt extract + varying volumes of *N*/10 NaOH and water to total volume 40 cc. The mixtures were incubated at 37° for 14 hours. After withdrawing 10 cc. for the determination of p_H , the remaining 30 cc. were subjected to the formaldehyde titration. The controls were performed on malt extract + water, at the same values of p_H , and a control for edestin alone.

Table IX.

p_H of reaction mixture	4.0	4.4	4.6	4.9	5.1	5.5
Proteolysis, cc. <i>N</i> /10 NaOH (controls deducted)	2.65	2.80	2.75	2.70	2.55	1.50

Exp. 10. Edestin. Proteolysis in presence of acetate. The process was exactly as in Exp. 9 except that acetic acid was used instead of HCl. There was comparatively little protein in suspension in the reaction mixture, but a little was unavoidable in some cases.

Table X.

p_H of reaction mixture	4.25	4.40	4.64	4.82	5.05
Proteolysis, cc. <i>N</i> /10 NaOH (controls deducted)	1.65	1.40	1.35	1.25	1.20

Exp. 11. Edestin. Electrometric titration. Table XI gives the volumes of *N*/10 NaOH added to 0.05 g. of edestin, and the p_H resulting. The protein was dissolved in *N*/10 HCl.

Table XI.

<i>N</i> /10 NaOH cc.	4.0	4.2	4.4	4.5	4.6	4.7	4.8	4.9
p_H	2.41	3.37	3.54	4.04	4.47	4.82	6.58	8.58
$\Delta A/\Delta p_H$		0.21	1.18	0.20	0.23	0.28	0.06	0.05

Exp. 12. Fibrin. Kinetics. 2 g. of fibrin were dissolved in *N*/5 NaOH with heat, and brought back to p_H 6.8 and 4.6 in two portions. There was no precipitation at p_H 6.8, but precipitation took place at p_H 4.6. *N*/2 acetic acid was used for the purpose. Two experiments were performed, one on each solution, using 50 cc. of fibrin + 30 cc. of malt extract + water to 130 cc. Controls were treated similarly, using boiled malt extract. The reaction was checked by withdrawing 20 cc. at intervals, adding the required volume of *N*/2 acetic acid to bring to maximum precipitation and filtering. Total nitrogen was determined on the filtrates.

Table XII.

Time (hours)	1	2	4	
Action, cc. <i>N</i> /10 (controls deducted)	0.5	1.2	1.5	At p_H 4.5
	—	0.6	0.8	At p_H 6.9

Exp. 13. Fibrin. Proteolysis at different reactions. 5 g. of fibrin + 50 cc. of *N*/2 acetic acid were heated to just below boiling, allowed to stand for a time in the cold, filtered and the swollen fibrin dissolved in 300 cc. of *N*/8 NaOH by heating. By this means the fibrin was dispersed with less drastic treatment than in the previous experiment. It still precipitated, however, at p_H 4.8 on neutralisation, instead of p_H 6.8 which is the isoelectric point of fibrin. 20 cc. of fibrin + 15 cc. of malt extract + the required volumes of acetic acid or sodium hydroxide + water to total volume 80 cc. After 5 hours at 46°, 40 cc. were withdrawn and brought to p_H 4.8 by addition of the necessary volumes of acetic acid or NaOH, etc. as in Exp. 12.

Table XIII.

p_H of reaction mixture	3.81	4.20	4.58	5.00	6.03	7.59	8.27	9.00
Proteolysis, cc. <i>N</i> /10 HCl (controls deducted)	2.3	0.9	1.1	1.6	2.3	0.6	0.3	0.0

Exp. 14. Fibrin. Electrometric titration. The fibrin was dispersed as in Exp. 12. Table XIV gives the volume of *N*/10 HCl added to 0.1 g. of fibrin in *N*/5 NaOH. The buffer coefficients are also given.

Table XIV.

<i>N</i> /10 HCl cc.	9.0	9.3	9.5	9.6	9.8	9.9	10.0	10.2	10.4
p_H	7.60	6.22	5.91	5.46	4.86	4.16	3.80	3.11	2.89
$\Delta A/\Delta p_H$		0.22	0.64	0.22	0.33	0.14	0.28	0.29	0.91

Table XV. *Summary of p_H values corresponding to maximum values of buffer coefficients and proteinase activity.*

Protein	p_H range of maximum	
	Buffer coefficient	Activity of proteinase
Egg-albumin	3.31-3.67 (present communication)	3.3-3.6
	3.40-3.53 [Hendrix and Wilson, 1928]	
	3.10-3.50 [Loeb, 1921]	
Caseinogen	3.00-3.35	3.0-3.35 and 5.57
	and	
	6.31-6.47 (present communication)	
	3.10-3.41	
Edestin	and	4.25-4.4
	6.10-6.52 [Loeb, 1921]	
	3.37-3.54 (present communication)	
Fibrin	3.30-3.60 [Hitchcock, 1922]	3.81 or less and 6.0
	3.11-4.16 (present communication)	
	and	
	5.91-6.22	

SUMMARY.

1. The proteinase of green malt acts at 46° optimally on crystalline egg-albumin at p_H 3.3 to 3.6, on caseinogen at p_H 3.35 and at 5.57, on fibrin at p_H 3.8 (or less) and at 6.0, and on edestin at 37° optimally at p_H 4.3.
2. These values of p_H approximate in most cases very closely to those of the maximum buffering power of the protein measured at 18°.
3. The proteinase appears to act on ionised protein on both sides of the isoelectric point, but there is no clear evidence that it is specific for zwitterions, although this is quite possible in the cases of those proteins in which zwitterions exist.

REFERENCES.

- Clark (1928). The determination of hydrogen ions. 3rd edit., p. 214. (Baillière, Tindall and Cox.)
- Hendrix and Wilson (1928). *J. Biol. Chem.* **79**, 389.
- Hitchcock (1922). *J. Gen. Physiol.* **4**, 597.
- Hopkins (1929). *Biochem. J.* **23**, 1022.
- and Burns (1930). *J. Inst. Brew.* **35**, 9.
- Linderstrøm-Lang and Sato (1929, 1). *Compt. Rend. Trav. Lab. Carlsberg*, **17**, No. 17.
- — (1929, 2). *Z. physiol. Chem.* **184**, 83.
- Loeb (1921). *J. Gen. Physiol.* **3**, 85, 547.
- Luers and Malsch (1929). *Woch. Brau.* **46**, 265.
- Michaelis and Pechstein (1912). *Biochem. Z.* **47**, 260.
- Michaelis and Szent-Györgyi (1920). *Biochem. Z.* **103**, 178.
- Mill and Linderstrøm-Lang (1929). *Compt. Rend. Trav. Lab. Carlsberg*, **17**, No. 10.
- Willstätter, Grassmann and Ambros (1926). *Z. physiol. Chem.* **151**, 307.

XXXIII. CAROTENE AND VITAMIN A. THE CONVERSION OF CAROTENE INTO VITAMIN A BY FOWL.

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It has now been established with a fair degree of certainty that carotene possesses vitamin A activity [Euler, Euler and Hellström, 1928; Moore, 1929, 1, 2, 1930; Collison, Hume, Smedley-MacLean and Smith, 1929; Kawakami and Kimm, 1929; Hume and Smedley-MacLean, 1930; Karrer, Euler and Rydholm, 1930]. It has also been shown that, in the rat, carotene is converted into the "classical" vitamin A of liver oils, characterised by the blue colour given with the antimony trichloride reagent (absorption band $610-630\mu\mu$) and by the presence of an absorption band in the region of $325\mu\mu$ [Capper, 1930; Moore, 1930]. Before any generalisations could be made it seemed desirable that similar experiments should be carried out with animals differing widely from the rat. For this reason, as well as for the fact that in the fowl the problem presents points of peculiar interest, the work to be described was undertaken.

It will be shown that the fowl as well as the rat can convert carotene into vitamin A; that the vitamin A requirements of the fowl are higher than those of the rat weight for weight, and that the liver oil is normally very much richer in vitamin A than is cod-liver oil.

EXPERIMENTAL.

Exp. 1. The pullets used were White Wyandottes which had originally been intended for another experiment, and a deficiency of vitamin A in the diet was in the nature of an unexpected accident. The basal diet consisted of:

Cereals

Bran 2 parts, pollards 1 part, yellow maize meal	
1 part, Sussex ground oats 1 part by weight...	78 %
Soya bean meal	18 %

Mineral mixture

Steamed bone flour	10 parts by wt.	
Commercial potassium chloride ("muriate of potash")	2	"
Common salt	1.5	"
Sulphur	0.25	"
Iron oxide	0.1	"
Potassium iodide	0.01	" 4 %

A scratch grain consisting of whole wheat was fed at the rate of approximately 50 % of the total food consumed and oyster shell was supplied *ad lib.* as a source of lime.

Group A had been kept indoors in a hut glazed with vita glass and had received the basal diet only.

Group B had received the basal diet but in addition had had free access to an open air grass run.

Group C had been kept indoors in a hut glazed with ordinary glass and in addition to the basal diet had received natural cod-liver oil at the rate of about 2 % of their diet.

The birds had been put on these diets as day-old chicks, except that for the first 9 weeks separated milk replaced the mineral mixture. At the age of approximately 16 weeks while the birds of Groups B and C remained healthy and normal the majority of those of Group A began to decline in weight and were obviously unwell. Their sense of balance appeared to be disturbed and they walked with difficulty and with a staggering gait. Rachitic trouble was at first suspected but normal calcium and phosphorus figures were obtained by Mr R. H. Common for samples of their blood-serum, and when two of the birds were killed autopsy revealed normal ossification. On examination of their livers for the presence of vitamin A negative results were obtained both by the SbCl_3 test, and spectroscopically by the absence of any selective absorption in the region of 325μ . The liver oils of birds of Groups B and C and of birds procured in the ordinary market all gave an intense blue colour with SbCl_3 and a well marked absorption band in the region of 325μ .

In testing the livers for vitamin A the technique described by Moore [1930] was followed in the main. The liver was minced, mixed with about twice its bulk of 5 % KOH and left for several days at least. It was then extracted four times with ether, the ethereal extract washed three times with water, dried over anhydrous sodium sulphate and the ether evaporated off by slight warming under reduced pressure. The oil thus obtained was then dissolved in chloroform in such dilution that 0.2 cc. solution + 2 cc. SbCl_3 reagent gave between 3 and 10 blue units on a Lovibond tintometer. The "blue units" and "yellow units" were then calculated as described by Moore [1930].

The apparatus used for the determination of the absorption spectra

was that previously described [Capper, 1930]. The results are shown in Table I.

Table I.

No. of bird	Diet	Wt. of liver (g.)	Wt. of liver oil obtained (g.)	Total "blue units" found in liver	"Blue units" per g. of liver	Total "yellow units"
A 1	Basal only	11	0.15	0	0	—
A 2	"	18	0.10	0	0	15
B 1	Basal and greenstuff	16	0.22	2,200	140	150
B 2	"	24	0.19	48,100	2,000	6500
C 1	Basal and cod-liver oil	24	0.13	24,800	1,030	80
*C 2	"	23	0.13	5,500	240	25
M 1	Birds procured in the open market	43	1.05	103,000	2,370	1060
M 2		33	0.90	41,200	1,250	375
M 3		27	0.50	375,000	10,100	—

* This value is probably too low as there was some loss due to emulsification in certain stages of the separation.

The large amount of vitamin A that may sometimes be found in the livers of fowls is shown by the values recorded for the liver of hen No. M 3 where the natural liver oil appears to be richer in vitamin A than the unsaponifiable residue of cod-liver oil, which generally gives about 300,000 blue units per g. of concentrate as compared with 750,000 blue units per g. of this liver oil. The high vitamin A content indicated by the SbCl_3 test was confirmed by the persistence of the absorption band in the region of 325μ in the absorption spectrum and for M 1 by feeding tests on rats carried out at Cambridge by Dr Moore, who found the liver oil active as vitamin A at about the level indicated by these figures (see Fig. 5).

Nine birds of Group A were then selected for further experiment. All were confined in the same wooden house glazed with vita glass; three continued to receive the basal diet only, three received in addition cod-liver oil concentrate, while the remaining three received the basal diet + carotene dissolved in arachis oil. The cod-liver oil and carotene solutions were administered orally by means of a fountain pen filler. The growth curves and daily doses of these birds are shown in Fig. 1.

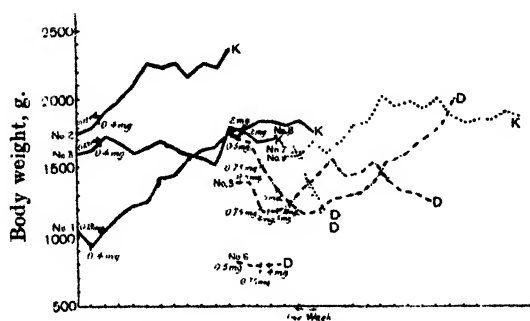


Fig. 1. ——— Birds receiving carotene.
 - - - - - " " cod-liver oil concentrate.
 " " neither carotene nor cod-liver oil.
 The figures indicate the daily doses. D=died. K=killed.

The large vitamin A requirement of fowls was not realised at first and the birds continued to decline in weight and to become more helpless until the daily dose of cod-liver oil concentrate had been increased to about 10 mg. and of carotene to 0.5 mg. (The carotene used was prepared by Dr Moore from red palm oil, M.P. 168° (uncorr.).) Before the dose of carotene had been increased to 0.5 mg. No. 1 had so declined as to be unable to stand upright and lay helpless on its side; with the increased dose of carotene, however, recovery was most rapid, walking became possible within 24 hours and weight steadily increased. No. 4 had reached almost the same state before the dose of cod-liver oil concentrate had been increased to 10 mg. daily, after which recovery and growth were continuous and death after 16 weeks was due, not to any dietary deficiency, but to prolapsus of the oviduct. Nos. 5 and 1 eventually became broody and for this reason lost weight during the final weeks of the experiment. Of the control birds which continued to receive the basal diet only, Nos. 7 and 8 rapidly declined and died, No. 7 developing eye trouble resembling xerophthalmia. No. 9, which was the strongest bird at the beginning of the experiment, continued to live and at no time exhibited any pathological condition. Since the birds were confined together it may conceivably have received some vitamin A from the excreta, etc. of the birds receiving cod-liver oil concentrate or carotene or it is possible that it possessed lower requirements and utilised what it received more efficiently.

On examination the liver oils of all the birds that had received either cod-liver oil concentrate or carotene gave positive reactions for vitamin A with SbCl_3 and showed absorption bands at about 325μ , while the oils of the birds that had received the basal diet only invariably gave negative results both colorimetrically with SbCl_3 and spectroscopically. The results of the examination of the livers are shown in Table II.

Table II.

No. of bird	Daily dose during final weeks of exp.	Wt. of liver (g.)	Wt. of liver oil (g.)	Total "blue units" with SbCl_3 found in liver	"Blue units" per g. of liver	Total "yellow units" in CHCl_3
1	2 mg. carotene	32	0.24	8,350	265	20
2	1 mg. "	56	1.63	330	6	150
3	2 mg. "	23	0.28	3,700	160	150
4	10 mg. C.L.O. conc.	36	0.46	8,250	225	25
5	10 mg. "	20	0.18	10,500	525	25
6	4 mg. "	32	0.16	165	5	10
7	0	34	0.14	0	0	5
8	0	26	0.14	0	0	10
9	0	41	0.35	0	0	—

This experiment suggests strongly that the fowl as well as the rat can transmute carotene into vitamin A. At the same time it is in some respects open to criticism, and not completely satisfactory. Since the fowls used were approaching maturity, regular growth curves could not be expected; the diet was not sufficiently synthetic and birds receiving different diets were confined

in the same house. For these reasons a second experiment was carried out on more rigid lines.

Exp. 2. The basal diet used had the following composition:

Caseinogen (Glaxo physiological AB)	...	18 %
Agar	2
Yeast (dried)	15
Dextrin	60
Salt mixture	5

The salt mixture had the composition given by Hart, Halpin and Steenbock [1920].

Vitamin D was supplied by administering 2 drops (afterwards increased to 10 drops) of radiostol or an equivalent amount of ostelin daily. The food was supplied dry and drinking water was available *ad lib.* No litter was used but sand covered the floor of the houses.

Twelve White Wyandotte¹ chickens (Group A) were placed on this diet at the age of 6 weeks and confined in a wooden poultry house. Six more chickens (Group B) from the same batch were placed in a similar house and received the same basal diet with the addition of 0.5 mg. cod-liver oil concentrate daily.

For some weeks the Group B birds gained weight considerably faster than the others, as may be seen from the growth curves in Figs. 2 and 3, but after about 6 weeks both groups declined in weight and four of Group A and three of Group B died. (One bird in Group A died soon after being placed on the diet and has been omitted from consideration.) When examined for the presence of vitamin A the livers of all six gave negative results. It was then realised that the doses of cod-liver oil concentrate were insufficient and these were increased as shown in Fig. 2. It is interesting to note that only No. 7 exhibited eye trouble in any way resembling xerophthalmia, nor did any of them display the curious staggering walk shown by the birds in Exp. 1. The "third eyelids" of all the birds were partially closed and they had a generally dejected appearance. *Post mortem* examination of those that died revealed powdery white deposits which gave the murexide test for urates round the heart, liver and other organs, and the disease was diagnosed in the Animal Diseases Laboratories of the Northern Ireland Ministry of Agriculture as visceral gout. This condition would appear to be similar to, but more severe than, that found by Hart *et al.* [1924] in their work on the nutritional requirements of chicks.

The remaining birds of Group A were now given carotene dissolved in arachis oil. The daily doses and growth curves are shown in Fig. 3.

With Nos. 7, 10 and 13 the disease had progressed too far and they succumbed within 2 days. All the remaining birds in both groups now rapidly

¹ In a previous communication these chickens were by a slip reported as White Leghorns (Capper, *Nature*, 1930, 126, 685). The opportunity is now taken to correct the error.

improved in appearance and at once began to increase in weight. Eventually Nos. 0, 2, 6 and 17 became fully mature birds, and when killed ossification was found to be good and, except for a certain paleness, the organs appeared

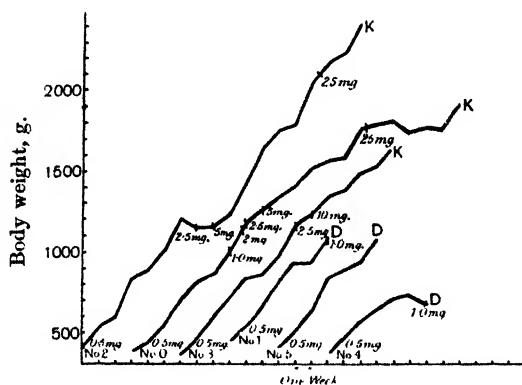


Fig. 2. Growth curves of birds receiving cod-liver oil concentrate. The figures indicate the daily doses. D=died. K=killed.

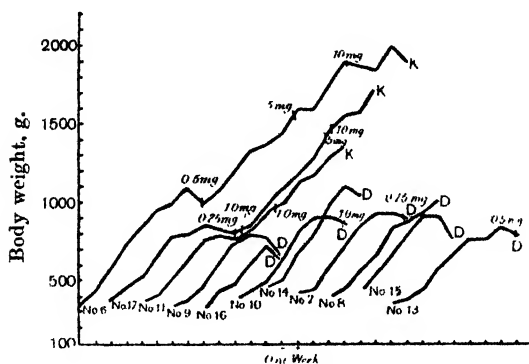


Fig. 3. Growth curves of birds receiving carotene and controls. The figures indicate the daily doses. D=died. K=killed.

normal and fully developed. No. 6 commenced to lay at the age of 23 weeks, having then been fed for 17 weeks on the synthetic diet and in the next month which followed before it was killed laid 20 eggs ranging in weight from 34.5 g. to 59 g. with an average weight of about 40 g. The shells of these eggs were brown in colour but the yolks were almost colourless, possessing only a faint yellow tinge when hard boiled.

The livers of all the birds were examined for the presence of vitamin A both colorimetrically by SbCl_3 and spectroscopically. In addition, representative liver oils were despatched to Cambridge in evacuated tubes and tested biologically for vitamin A on rats by Dr Moore. The results of the examination of the liver oils are shown in Table III.

Table III.

No. of chicken	Diet during final weeks	Wt. of liver (g.)	Wt. of liver oil (g.)	Total "blue units" found in liver	"Blue units" per g. of liver	Total "yellow units" in liver	Absorption spectrum
0	Basal + 25 mg. C.L.O. conc.	42	0.77	8,250	190	50	Band at 325 μ
1	Basal + 0.5 mg. C.L.O. conc.	30	0.22	0	0	8	No band near 325 μ
2	Basal + 25 mg. C.L.O. conc.	63	0.59	15,100	240	30	Band at 325 μ
3	Basal + 10 mg. C.L.O. conc.	34	0.23	11,000	320	15	—
4	Basal + 0.5 mg. C.L.O. conc.	22	0.16	0	0	8	No band near 325 μ
5	Basal + 0.5 mg. C.L.O. conc.	25	0.19	0	0	15	—
6	Basal + 10 mg. carotene	41	0.59	8,200	200	100	Band at 325 μ
7	Basal only	20	0.12	0	0	10	No band near 325 μ
8	"	29	0.16	0	0	10	"
10	"	25	0.13	0	0	10	No band near 325 μ
11	Basal + 1 mg. carotene	28	0.24	250	9	29	—
13	Basal only	22	0.14	0	0	8	No band near 325 μ
14	"	24	0.09	0	0	15	"
15	"	22	0.21	0	0	—	—
16	"	17	0.08	0	0	10	No band near 325 μ
17	Basal + 10 mg. carotene	44	0.19	1,300	30	35	Band at 325 μ

The liver oils of chickens which had received the same diet gave similar absorption spectra. Typical examples of the absorption spectra of the liver oils are shown in Fig. 4, and in Fig. 5 are shown the growth curves and daily doses of the rats which were given the chicken-liver oils as a source of vitamin A.

It may be pointed out here that when the chickens were first placed on the synthetic diet, after having received a normal diet containing greenstuff, their beaks and shanks were highly pigmented, but after a few weeks on the carotenoid-free synthetic diet the yellow colour faded completely and even after receiving large doses of carotene daily for periods of up to 9 weeks no increase in pigmentation was noted, which is in agreement with the results obtained by Palmer and Kempster [1919] who found that xanthophyll was necessary for the pigmentation of the beak and shanks. In addition, while the liver oil of a hen on a normal diet was found to give deeply pigmented solutions in which absorption bands corresponding to those recorded for xanthophyll were observed, the liver oils of the birds which had received the synthetic diet + carotene gave solutions which were only slightly yellow in colour.

If we take 0.5 mg. carotene as the minimum daily requirement for a hen weighing 2000 g. and compare it with the minimum daily requirement of carotene (0.002 mg.) found by Moore [1930] for a rat weighing 100 g. it is

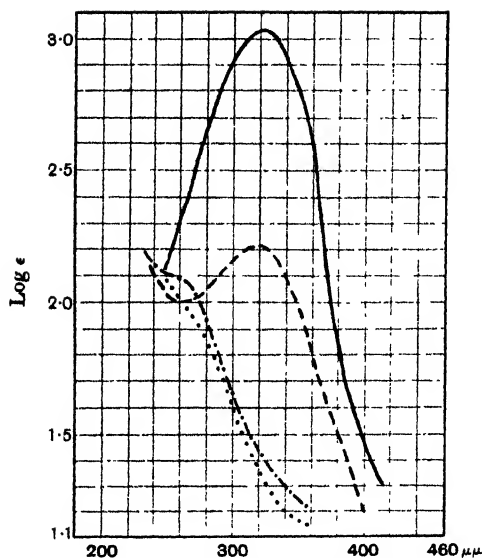


Fig. 4. — Liver oil of chicken No. 3 in chloroform.

— " " 6 "
 - - - " " 8 "
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ϵ is defined by $\log I_0/I = \epsilon cd$, where c is the concentration of the liver oil in g. per cc., d is the cell thickness in centimetres.

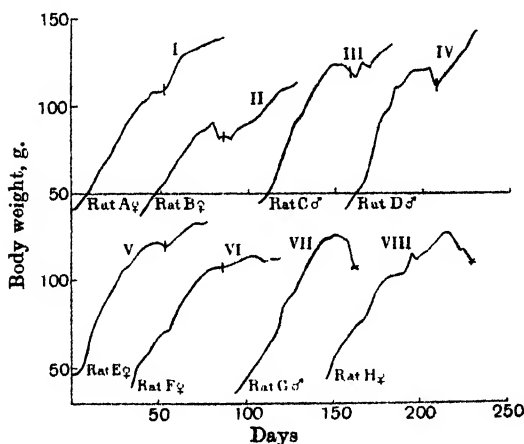


Fig. 5. Confirmation of the vitamin A activity of chicken-liver oils by rat-growth tests.

I and II. Liver oil of bird M 1 (Table I) 0.013 mg. daily = 1 B.U.

III. Liver oil of bird 3 (Table II) 0.2 mg. daily = 2 B.U.

IV. " " bird 6 " (Table III) 0.4 " = 4 B.U.

V. " " bird 6 " (Table III) 0.4 " = 5 B.U.

VI. " " bird 11 (Table III) 2.0 " = 2 B.U.

VII and VIII. Negative controls.

Rats A, B, E, G received a basal diet containing Glaxo caseinogen 20%, rice starch 60%, palm kernel oil 15%, salt mixture 5%, supplemented by 10% of dried yeast and one drop of radiostol daily. The basal diets of rats C and D differed in containing a similar amount of arachis oil in place of palm kernel oil and 7.5% of marmite extract in place of dried yeast, the basal diets of rats F and H in containing additional starch in place of the fat component. The test doses of chicken-liver oils were diluted in arachis oil and administered at the points indicated in the figure by a vertical line.

seen that the vitamin requirements of the fowl appear to be greater than those of the rat weight for weight.

As misleading results have been obtained in the past by other workers through the use of a solvent in which the carotene altered, it may be stated that the solution of carotene in arachis oil used in the experiments described was kept in a cool dark place when not in use and no decomposition of the carotene was detected when the depth of colour of the solution after some months was measured in a Lovibond tintometer and compared with the colour of a freshly prepared solution.

DISCUSSION.

These experiments support the general theory that in animals carotene behaves as a precursor of vitamin A as suggested originally by Moore [1929, 3]. Even with large doses of carotene the liver oils were only slightly more pigmented than when no carotene was given, but while the liver oils of the fowls fed on the vitamin A-free diet gave no blue colour with the antimony trichloride reagent, no absorption band at $325\mu\mu$ and were biologically inactive, those of fowls given carotene in addition gave an intense blue colour (absorption band $610\text{--}630\mu\mu$) with SbCl_3 , an absorption band at $325\mu\mu$ and proved adequate as a source of vitamin A when fed to rats.

The result of recent work on the relation of carotene to vitamin A makes it clear that biological tests alone cannot distinguish between carotene and the "classical" vitamin A and it would seem probable that vitamin A is a product of animal synthesis and ultimately owes its origin entirely to carotene. Land animals can obtain carotene from vegetable matter, while Ahmad [1930] has shown that carotene in diatoms is probably the source of the vitamin A of fish-liver oils.

SUMMARY.

Chickens were successfully reared to maturity on a synthetic vitamin A-free diet to which either carotene or cod-liver oil concentrate was added. The carotene was not stored in the liver unchanged but was converted into vitamin A characterised by the blue colour given with SbCl_3 (absorption band $610\text{--}630\mu\mu$) and the presence of an absorption band at $325\mu\mu$.

The beaks and shanks of chickens, which had become colourless through the absence of carotenoids from the diet, did not become more yellow when carotene was added to it.

The poultry disease known as visceral gout would appear to be related to vitamin A deficiency and to be curable by the administration either of carotene or of cod-liver oil.

The vitamin A content of hen-liver oils is shown to be very high and the vitamin A requirements of the fowl large.

We have pleasure in expressing our thanks to Dr Moore for supplying the carotene used in the experiments, for carrying out the biological tests recorded

and for his unceasing interest in the work; to Imperial Chemical Industries, Ltd., for a grant which helped to defray the cost of the experiments; to Messrs Joseph Nathan, Ltd., for generously supplying the ostelin and cod-liver oil concentrate used, and to Miss A. C. Woods and Miss H. Kennedy of the Agricultural Research Institute of Northern Ireland at Hillsborough, without whose skilful care of the fowl the experiments would have been impossible.

REFERENCES.

- Ahmad (1930). *Biochem. J.* **24**, 860.
Capper (1930). *Biochem. J.* **24**, 980.
Collison, Hume, Smedley-MacLean and Smith (1929). *Biochem. J.* **23**, 634.
Euler, Euler and Hellström (1928). *Biochem. Z.* **203**, 370.
Hart, Halpin and Steenbock (1920). *J. Biol. Chem.* **43**, 421.
Hart, Steenbock, Lepkovsky and Halpin (1924). *J. Biol. Chem.* **60**, 341.
Hume and Smedley-MacLean (1930). *Lancet*, i, 290.
Karrer, Euler and Rydberg (1930). *Helv. Chim. Acta.* **13**, 1059.
Kawakami and Kimm (1929). *Proc. Imp. Acad. Tokyo*, **5**, 213.
Moore (1929, 1). *Biochem. J.* **23**, 803.
—— (1929, 2). *Biochem. J.* **23**, 1267.
—— (1929, 3). *Lancet*, ii, 380.
—— (1930). *Biochem. J.* **24**, 692.
Palmer and Kempster (1919). *J. Biol. Chem.* **39**, 331.

XXXIV. VITAMIN A AND CAROTENE.

VII. THE DISTRIBUTION OF VITAMIN A AND CAROTENE IN THE BODY OF THE RAT.

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IN a preceding communication of this series [Moore, 1930] it was shown that the feeding of excess of carotene to albino rats resulted in an accumulation in the liver of large amounts of the colourless vitamin A, and the inference was drawn that the pigment, or some part of it, is the parent substance of the vitamin, capable of conversion in the normal course of metabolism. In this work attention was focused on the liver on account of its importance in the storage of the vitamin, and no attempt was made to examine other organs, or to decide whether the conversion of carotene is effected in the alimentary tract or after absorption into the blood stream. It seemed desirable, therefore, to make a detailed study of the distribution of vitamin A and carotene throughout the body and intestinal contents of rats receiving large amounts of carotene, in the hope of gaining further knowledge on these points.

Since Palmer and Kennedy [1920] have shown that carotene does not appear in the body fat of the rat even when large amounts are introduced into the diet, it is natural that the question of the distribution of the pigment in this animal should have hitherto attracted little attention. The question of vitamin A distribution, on the other hand, has been studied by numerous workers, notably Sherman and Boynton [1925], who used the rat-growth technique in estimating the vitamin, and by Kerppola [1930], who has employed the antimony trichloride reaction. Using tissues from rats deriving their supplies of vitamin A from the inclusion in their diet of 33½ % of dried milk powder, the American workers found that daily doses of 0.02 g. liver, 0.1 g. lung, 0.1 g. kidney, or 4.0 g. of muscle were necessary to promote growth in other rats, and it was deduced that under these nutritional conditions nine-tenths of the vitamin A reserves of the body (neglecting those of the adipose tissue and skin) were to be found in the liver. The results of Kerppola, although not reduced to quantitative terms, suggest a similar distribution in regard to the liver and lungs. A positive reaction was also obtained from the intestines and contents, but negative results were given by all other organs examined. The predominance of the liver in the storage of the vitamin has

¹ In the whole time employment of the Medical Research Council.

also been reported in many other animals in addition to the rat; mention may be made of the work of Rosenheim and Webster [1927] on the fulmar petrel, and the comparison by Ahmad and Drummond [1930] of the vitamin A activities of the liver and body oils of fishes.

EXPERIMENTAL.

The experimental procedure was designed to determine the relative distributions of the pigment and vitamin under conditions in which the former was supplied in amounts greatly in excess of the immediate physiological requirements. Albino rats received synthetic diets in which excess of carotene was included, for reasons of convenience and economy, as red palm oil or, in one instance, as carrot-fat. After receiving these diets for periods sufficiently prolonged to ensure that a large superfluity of pigment had been made available certain of the rats were killed by coal gas and examined without further treatment. Others, with a view to the removal of traces of unaltered carotene that might have been present in the blood stream, were transferred to a diet deficient in vitamin A for appropriate periods before killing.

In estimating the amounts of vitamin A and carotene in the various tissues the methods employed were essentially those previously adopted in the extraction and colorimetric examination of liver oils [Moore, 1930]. Some modification, however, was necessary in the procedure adopted in the interpretation of results, since it was found that in red palm oil and carrot-fat the ratio of the natural yellow value to the blue value given in the antimony trichloride reaction was not more than about 4 to 1, as compared with the value of 11 to 1 previously quoted for pure carotene. Although at first sight this low ratio might be taken to imply the presence of carotene and vitamin A simultaneously, it is actually most improbable that this should have been the case, since no evidence of absorption at $610-630\mu\mu$, the position characteristic of vitamin A, could be detected when the blue colorations produced by these materials in the $SbCl_3$ reactions were examined spectroscopically. Two alternative explanations of the discrepancy may be put forward. In the first place since determinations of both yellow and blue values by the tintometer technique are subject to large experimental errors, the ratio between them becomes subject to a much greater error, so that the difference observed between the ratios may actually be less significant than it appears. Secondly it is probable that the yellow value of carotene, under certain natural conditions¹, may deteriorate in much the same way as it does under the action of benzoyl peroxide [Moore, 1929] leaving the blue value unchanged. In interpreting the present results confusion will be avoided by basing comparisons upon the yellow/blue ratios actually determined in the red palm oil and carrot-fat.

¹ Evidence of such deterioration has been obtained in the course of experiments in collaboration with Dr Woolf on the aerobic incubation of carotene with tissue preparations.

The distribution of vitamin A and carotene in rats receiving diets rich in carotene.

Rat 1, ♀ (118 g.). Carrot-fat diet (results shown in Table I). After receiving a diet deficient in vitamin A for 29 days this rat was cured by the administration of carrot-fat, admixed with the diet, at the level of about 250 mg.

Table I.

Organ	Wet weight (g.)	Weight of fat (g.)	Yellow units	Blue units	Inference
<i>Rat 1, ♀ (118 g.). Carrot-fat diet.</i>					
Stomach	3.1	0.048	200	250	Carotene?
Small intestine and contents	3.5	0.055	100	100	Carotene?
Large intestine and contents	3.1	0.147	10,000	3,500	Carotene
Liver	9.1	0.063	280	2,500	Vitamin A
Brain	1.1	0.018	0	0	—
Heart	0.7	0.005	0	0	—
Kidneys	1.5	0.008	0	0	—
Lungs	1.4	0.0111	0	0	—
Pancreas	0.05	0.008	0	0	—
Spleen	0.7	0.0074	0	0	—
Suprarenals	0.046	0.006	0	0	—
Thymus	0.8	0.0054	0	0	—
Intraperitoneal fat	2.7	0.49	0	0	—
<i>Rat 2, ♂ (370 g.). Red palm oil diet.</i>					
Alimentary tract and contents	13	1.05	2,500	375	Carotene
Liver	15	0.274	250	70,000	Vitamin A
Carcass	342	71.4	140	160	?
<i>Rat 3, ♀ (197 g.). Red palm oil diet.</i>					
Small intestine and contents	6.2	0.1664	360	100	Carotene
Liver	10.2	0.1616	980	70,000	Vitamin A
Lungs	1.6	0.0268	0	15	Vitamin A
<i>Rat 4, ♀ (208 g.). Red palm oil diet.</i>					
Liver	7.6	0.1395	570	80,000	Vitamin A
Blood	4.6	0.0195	1	0	—
Kidneys	2.2	0.0886	1	10	Vitamin A?
Lungs	1.3	0.0318	1	10	Vitamin A?
Intraperitoneal fat	11.4	9.84	20	150	Vitamin A

daily. The carotene content of the latter was equivalent to 40 natural yellow units and 15 SbCl_3 blue units per mg. The yellow/blue ratio in the ingested fat was therefore about 2.7, and the daily intake of carotene about 20 mg. Carotene feeding was continued for 37 days. Upon *post mortem* examination positive SbCl_3 reactions were given by the fats derived from the stomach, small intestine, large intestine, with their respective contents, and by the liver, negative reactions by the fats from the remaining organs and also by the intraperitoneal fat. In the fat from the large intestine a yellow/blue ratio of about 3 was given, suggestive of unchanged pigment, and this conclusion was supported by the observation of an absorption band at 590μ in the SbCl_3 reaction. The fats from the stomach and large intestine showed a yellow/blue ratio of about unity, but since the characteristic band of vitamin A could not be detected in the SbCl_3 colorations it is probable that this low ratio

was essentially due to deterioration of the pigment, possibly on account of an abnormally long period of cold storage before examination. The liver oil contained a small amount of unconverted pigment, but the main chromogen present was vitamin A, as typified by a yellow/blue ratio of 0.1, together with absorption at $610\text{--}630\mu\mu$ in the SbCl_3 reaction.

It may be pointed out that the excess of carrot-fat administered to this rat was probably poorly tolerated, since the rate of growth at the time of killing was not rapid. The vitamin A content of the liver was not so high as in the cases described below, in which the feeding of a lower level of pigment was continued over longer periods.

Rat 2, ♂ (370 g.). Red palm oil diet (Table I). In the course of a separate experiment this rat had received for 119 days a synthetic diet in which vitamin A was included as 0.02 mg. of crystalline carotene daily; it was then transferred to a diet containing 15 % of red palm oil for a further period of 173 days.

The red palm oil employed had a natural yellow colour equivalent to 2.6 units per mg., and gave in the SbCl_3 reaction a value of 0.6 B.U. per mg. The yellow/blue ratio thus worked out at about 4.

For *post mortem* examination this rat was dissected into three portions: (1) the alimentary tract and contents, (2) the liver and (3) the rest of the carcase¹. The fat obtained from the alimentary tract was characterised by a yellow/blue ratio of about 6, indicating the persistence of unconverted pigment. In the liver a very high level of vitamin A was found, characterised by a yellow/blue ratio of about 0.003 and by the appearance in the SbCl_3 test of the usual $610\text{--}630\mu\mu$ absorption band. The carcase fat, on the other hand, gave only a faintly positive reaction, so transient that it was impossible to make spectroscopic measurements. The yellow/blue ratio, moreover, may in this case have been deceptive, since it is probable that the yellow value observed was rendered unduly high through the admission of particles of diet adhering to the skin. All that can be said with safety, therefore, is that the colour value of this fat, whether attributable to vitamin A or carotene, was of a very low order in comparison with the values given by the fats of the liver and alimentary tract.

Rat 3, ♀ (197 g.). Red palm oil diet (Table I). The preparatory feeding of this rat was exactly similar to that adopted in the previous case, except that the red palm oil diet was given for the slightly longer period of 273 days. Only the small intestine, liver, and lungs were examined, positive SbCl_3 reactions being given in all three cases. In the intestine fat a yellow/blue ratio of about 3.6 was observed, suggesting the persistence of unchanged pigment. The usual high concentration of vitamin A together with a small amount of unconverted pigment was found in the liver. The lung fat gave only a faint reaction,

¹ Solution was effected quite easily without even mincing by warming with 5 % aqueous KOH in the usual way, although heating had naturally to be continued for a somewhat longer period than was necessary in the case of smaller amounts of tissues.

which, from the absence of accompanying yellow pigmentation, may probably be ascribed to the vitamin.

Biological tests. As a check on the genuine character of the extremely high colour values given by the liver oils throughout the present experiments it was decided in this case to carry out parallel rat-growth tests, the results of which are shown in Fig. 1. The liver oil was effective at 0.005 mg. (the lowest dosage examined), and thus possessed a biological activity in good agreement with its colour value.

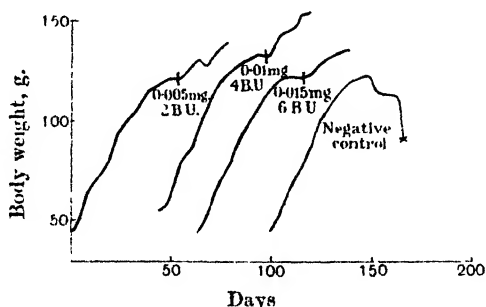


Fig. 1. Confirmation of the vitamin A activity of a representative rat-liver oil (that of rat 3, Table I) by rat-growth tests. The basal diet contained (Glaxo caseinogen 20%, rice starch 60%, palm kernel oil 15%, salt mixture 15%, supplemented by 10% of dried yeast and one drop of rachistod daily. The test doses were diluted in arachis oil and administered at the points indicated.

Rat 4, ♀ (208 g). *Red palm oil diet (Table I).* Preparatory feeding was again similar, the red palm oil diet being given for 278 days. The liver, kidneys, lungs, intraperitoneal fat and blood were examined, positive SbCl_3 reactions being given by all except the blood. The liver showed the usual high concentration of vitamin A, with small amounts of unconverted pigment. The kidneys, lungs and intraperitoneal fats all gave faintly positive reactions suggestive, in the absence of corresponding intense yellow coloration, of small amounts of vitamin A. The blood, which was obtained *post mortem* by cutting the blood vessels in the thoracic cavity just above the diaphragm, gave no indication of containing either carotene or vitamin A, although much of the sample taken must have flowed directly from the liver¹.

The distribution of carotene and vitamin A in rats restricted to a diet deficient in vitamin A after the preliminary feeding of excess of carotene.

Rat 5, ♂ (340 g). *Red palm oil diet, followed by vitamin A-free diet (Table II).* Preliminary feeding was similar to that adopted in the preceding cases, the red palm oil diet being given for 176 days. Before killing, a diet deficient in vitamin A was given for a further period of 10 days.

On *post mortem* examination a faintly positive reaction probably due to

¹ The result would indicate great efficiency on the part of the liver in removing carotene from the general circulation of the rat, which is in good agreement with its power of regulating the vitamin A concentration (see discussion).

the incomplete removal of traces of red palm oil was given by the fat of the small intestine. The fats from the stomach and large intestine gave reddish reactions, which can probably be ascribed to the presence of arachis oil in the vitamin A-free diet. In the liver a high concentration of vitamin A persisted together with small amounts of unconverted pigment. Of the remaining tissues the muscle and peritoneal fats gave faintly positive reactions suggesting the presence of vitamin A at a low concentration, while in all other cases negative results were obtained.

Table II.

Organ	Wet weight (g.)	Weight of fat (g.)	Yellow units	Blue units	Inference
<i>Rat 5, ♂ (340 g.). Red palm oil diet, followed by vitamin A-free diet.</i>					
Stomach and contents	2.2	0.0508	0	Reddish	?
Small intestine and contents	5.7	0.145	20	12.5	Carotene?
Large intestine and contents	3.0	0.15	1	Reddish	?
Liver	15.5	0.208	150	50,000	Vitamin A
Brain	2.3	0.019	0	0	—
Heart	1.1	0.0075	0	0	—
Kidneys	3.0	0.032	0	0	—
Lungs	2.4	0.025	0	0	—
Pancreas	0.78	0.015	0	0	—
Spleen	0.55	0.004	0	0	—
Suprarenals	0.04	0.004	0	0	—
Testes and seminal vesicles	1.93	0.0669	0	0	—
Thymus	0.85	0.007	0	0	—
Thyroids and parathyroids	0.6	0.0064	0	0	—
Muscles	117	3.9	10	40	Vitamin A
Intraperitoneal fat	23	12.2	18	120	Vitamin A
Bones	55	1.6	5	Reddish	?
Skin	76	13.0	40	Reddish	?

<i>Rat 6, ♂ (300 g.). Red palm oil diet, followed by vitamin A-free diet.</i>					
Small intestine and contents	5.0	0.5127	4	Reddish	?
Liver	14.2	0.1978	15	38,000	Vitamin A
Intraperitoneal fat	13.0	10.7	8	80	Vitamin A?

Rat 6, ♂ (300 g.). Red palm oil diet, followed by vitamin A-free diet (Table II). Preliminary feeding treatment was exactly as in the preceding case, but the final period on the vitamin A-free diet was now increased to 68 days. Only the small intestine and contents, liver, and intraperitoneal fat were examined. In the case of the small intestine a negative result was obtained. In the liver the concentration of vitamin A still remained at a high level, but no appreciable amounts of unconverted pigment were present. The intraperitoneal fat still gave a reaction suggesting the presence of vitamin A in relatively low concentration.

The excretion of carotene in the faeces of rats receiving excess of carotene.

Two samples of faeces were collected from the combined droppings of the rats receiving red palm oil, after the animals had received this diet for 131 and 234 days respectively.

The results obtained in both samples were very similar (Table III). It should be noted that in each case the yellow/blue ratio was about 6, pointing

to the presence of unconverted pigment. The concentration of pigment attained actually rose to quite a high level, representing, from calculations based on the SbCl_3 colour value, some 4 % of the faeces fat, or a 12-fold concentration over that of the ingested palm oil.

Table III.

Wet weight (g.)	Weight of fat (g.)	Yellow units	Blue units	Inference
<i>Sample of faeces collected after 131 days of red palm oil feeding.</i>				
1.54	0.097	4,500	750	Carotene
<i>Sample after 234 days.</i>				
7.3	0.458	23,000	3500	Carotene

The second sample of faeces fat was tested biologically as a source of vitamin A to rats, and was found active at a level of 0.25 mg. daily, which corresponds, on a basis of the SbCl_3 value, to a dosage of 0.01 mg. of actual carotene. The growth curves are shown in Fig. 2.

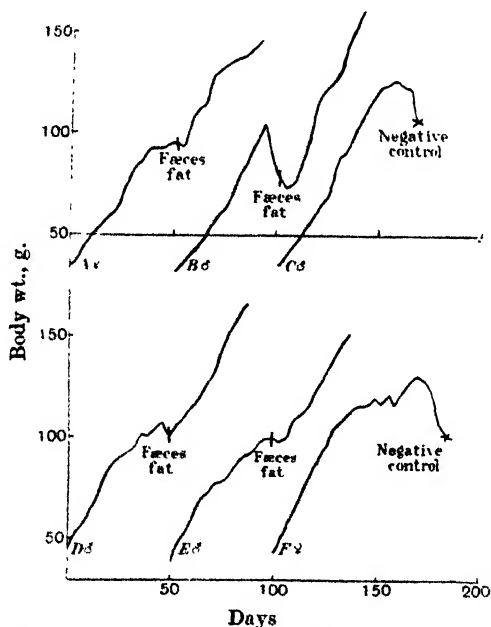


Fig. 2. The vitamin A activity of the faeces fat of rats receiving red palm oil. Rats *A*, *B* and *C* received a basal diet containing Glaxo caseinogen 20 %, rice starch 60 %, palm kernel oil 15 %, salt mixture 5 %, supplemented by 10 % of dried yeast and one drop of radiostol daily. Rats *D*, *E*, *F* received a diet differing only in the replacement of the palm kernel oil by additional rice starch. Doses of 0.25 mg. of faeces fat, equivalent to 0.01 mg. of carotene (1.8 B.U.) were administered in small amounts of arachis oil at the points indicated.

DISCUSSION.

The site of the conversion of carotene. A most consistent feature of the above results is the persistence of carotene, apparently unchanged, within the alimentary tracts of those animals which had received diets rich in carotene

up to the time of killing. It is obvious, therefore, that conversion to the vitamin is not effected in the course of digestion, but at some period subsequent to absorption into the blood stream. Since the liver is unique not only in its ability to hold large stores of vitamin A, but also in containing noteworthy amounts of unchanged pigment it would seem unnecessary to look beyond this organ for the site of the conversion of carotene. Further indirect evidence by other workers may be quoted in support of this view. Rydbom [1930] has carried out experiments on the fate of carotene when injected intramuscularly into the legs of rats, and has recovered *post mortem* substantial amounts of the pigment in apparently unchanged state from the tissues surrounding the site of injection. This result would at least suggest that the power to convert carotene to vitamin A is not possessed indiscriminately by all animal tissues. The special importance of the liver, however, is most strikingly illustrated by the work of Buckley *et al.* [1930], who have encountered cases of parenchymatous degeneration of the liver in cattle, presumably produced by the eating of poisonous plants, in which this organ becomes so overcharged with carotene that it appears deep yellow in colour, and is capable of yielding large amounts of crystalline carotene on suitable treatment. Since in the present writer's experience normal ox-liver oils have invariably been found to contain large amounts of vitamin A, accompanied by relatively small amounts of carotene (so small as to render their isolation almost impossible by means at present available), the above condition presents a pathological picture of the failure of carotene conversion coincident with a degeneration of the liver tissues.

The capacity of the liver in vitamin A storage. Apart from suggesting the probable site of the conversion of carotene, the present experiments are of interest in demonstrating the extremely high levels to which the vitamin A reserves of the liver may rise when lavish amounts of carotene are included in the food supply. In the case of the rat these levels may attain values quite out of proportion to immediate or future requirements. Thus in rat No. 2 (Table I) the liver was found to contain vitamin A equivalent to 70,000 blue units in the SbCl_3 test. If we assume that 2 B.U. per day would have represented the minimal requirements of this animal, then this store would have been adequate to have lasted for about a century, as compared with the natural life period of about 3 years.

Vitamin A in the remainder of the body. In contrast with the high level attained in the liver the amounts of vitamin A distributed throughout the remainder of the body are relatively small, although in some cases representing a supply sufficient for two or three months. In confirmation of Sherman and Boynton [1925] indications of the presence of vitamin A were observed in the fats obtained from the lungs and kidneys of certain animals (Nos. 3 and 4, Table I). The colour values obtained agree well with the minimal rat doses of the fresh tissues as determined by these workers, although, of course, the conditions of vitamin A excess were quite different. It must remain doubtful, however, whether these results should be taken to imply

that the organs concerned play any outstanding part in the metabolism of the vitamin. Not only were negative results given by the same organs in two other rats (No. 1, Table I and No. 5, Table II) but even in the cases under review the superiority of the vitamin A content over the general level in the stored fat of the body was so small as to be of very doubtful significance. Indeed from the aspect of quantity as opposed to concentration the stored fat (*e.g.* intraperitoneal fat) certainly contained the great bulk (at least 90 %) of the total vitamin A reserves found outside the liver. The consistently negative results given by the remaining organs of rats Nos. 1 and 5, while not necessarily implying that vitamin A was completely absent, must similarly suggest that the concentration of vitamin did not rise above that of the stored fat, which would not have given appreciably positive results if only minute quantities similar to those obtained from the various organs, had been available for testing.

The rôle of the liver in the regulation of the distribution of vitamin A. When an attempt is made to co-ordinate the conclusions reached in the two preceding paragraphs it appears that the liver must play a large part in the regulation of the concentration of vitamin A throughout the remainder of the body. This rôle, of course, is linked up with that of storage, but whereas the function of the liver in receiving and concentrating superfluous amounts of vitamin A from the diet has always been fully realised, its complementary function in facilitating the maintenance of a low level of vitamin A concentration in the body tissues has not been sufficiently appreciated. The case of rat No. 2 (Table I) provides a good illustration of the importance of this function. The concentration of carotene in the red palm oil included in the diet of this rat was equivalent to about 0.6 B.U. per mg., while in the liver oil the concentration of vitamin A was equivalent to about 250 B.U. per mg. If, for the sake of simplicity in argument, we compare these values directly, without making any allowance for the fact that different chromogens are involved, then the concentration of vitamin A in the liver oil may be considered to correspond roughly to an activity some 400 times greater than that of the ingested red palm oil. When on the other hand we turn to the fat derived from the remainder of the body (carcase fat) we find that the concentration of vitamin A is equivalent to not more than 0.002 B.U. per mg., which corresponds to only 1/300 of the activity in the ingested fat, or less than 1/100,000 of the activity of the liver oil. From these results it may be inferred that, while the vitamin A concentration in the liver may vary over an enormously wide range without producing any obvious effect, the concentration in the remainder of the body does not rise above a prescribed limit.

The efficiency of the conversion of carotene. In the previous communication [Moore, 1930] a point of difficulty arose in explaining how cod-liver oil concentrates, supposed by Drummond and Baker [1929] to contain only a minute proportion of actual vitamin A, could approach pure carotene so closely in

vitamin A activity. It was suggested either that the estimate of Drummond and Baker might be unduly low, or alternatively that the conversion of carotene might be of an inefficient character, thus necessitating its administration at a dosage greatly exceeding the amount actually converted into the vitamin.

It may be recalled that carotene gave a value in the SbCl_3 reaction of about 180 B.U. (at $590\mu\mu$), and was effective in growth tests at levels down to about 0.004 mg. A typical cod-liver oil concentrate, on the other hand, gave a colour value of about 270 B.U. (at $610\text{--}630\mu\mu$) and was found in one instance to be biologically active at 0.0033 mg., although 0.01 mg. was necessary to ensure regular results. These figures indicated that vitamin A must at least have a slightly higher colour value than carotene, but since it was by no means necessary to suppose that the different blue colorations given by carotene and vitamin A bore exactly the same relation to biological activity, this evidence did not seem incompatible with the simple assumption that the conversion of carotene to the vitamin might be almost complete, and that vitamin A might after all represent the main constituent of the cod-liver oil concentrates.

The data obtained in the present experiments, however, must now lead to a revision of this view, since in several cases the rat-liver oils themselves (not their unsaponifiable fractions) gave colour values much higher than those previously determined for cod-liver oil concentrates¹. Thus the liver oil of rats Nos. 3 and 4 (Table I) gave colour values of about 430 and 600 B.U. per mg. respectively. Preliminary experiments have indicated that the proportion of unsaponifiable matter in these oils is much greater than in cod-liver oils, but it is safe to assume that by saponification and the removal of sterols, etc. the colour values could be easily raised to a much higher level. Since there is every reason to believe that these colour values bear a genuine relation to the biological values of the oils (see Fig. 1) it must be inferred that preformed vitamin A not only possesses a much higher colour value than carotene, but also, under the usual conditions of administration, is biologically effective in much smaller doses.

Two alternative explanations of these findings might be advanced. In the first case it might be suggested that carotene is heterogeneous, containing in small amounts a component responsible for its biological and chromogenic activity, which is concentrated and converted to vitamin A in the liver. Secondly it might be supposed that the absorption and conversion of carotene is normally inefficient, but that actual conversion, when attained, is accompanied by a great increase in chromogenic value. The converted portion of the pigment, as vitamin A, now becomes available for utilisation by a second animal without a similar heavy loss, and the minimal dosage is therefore correspondingly reduced. This latter alternative is supported by the appearance

¹ The writer has been privileged to examine chicken-liver oils of approximately equal activity prepared by Mr N. S. Capper.

of excess of unchanged carotene in the faeces of the rats used in the present experiments, and for the present is perhaps to be preferred.

The effect of dietary vitamin A deficiency upon pre-existing reserves of vitamin A and carotene. The results obtained in the cases of rats Nos. 5 and 6 (Table II) are of interest in showing the effect of exposure to a diet deficient in vitamin A upon the reserves of vitamin A and carotene stored up during the period of excess. After receiving the deficient diet for periods of 10 and 68 days the vitamin A content of the liver oils remained extremely high. Similarly the intraperitoneal fats gave colour values differing but little from those shown by rats receiving carotene up to the time of killing. The most noteworthy change observed was the virtual disappearance of unchanged pigment from the liver oil of rat No. 6. The extremely low yellow/blue ratio (0.0004) shown by this oil supports almost to the degree of certainty the current view that pure vitamin A is completely colourless.

SUMMARY.

1. Albino rats were given diets containing lavish amounts of carotene either as red palm oil or carrot-fat for prolonged periods. The animals were then killed, and estimations of vitamin A and carotene were carried out on the dissected tissues by colorimetric methods.

2. Excess of carotene was found to persist apparently unchanged throughout the alimentary tract. The pigmented fat derived from the faeces was found to be biologically active at a level based upon its apparent carotene content.

3. The liver oils invariably contained vitamin A at extremely high concentrations, the oils themselves in several cases giving higher colour values than typical cod-liver oil concentrates. Small amounts of unconverted pigment were also present in the liver oils of all rats which had received carotene up to the time of killing.

4. Indications of the presence of vitamin A were usually shown by the "storage" fats of the body, the concentration per unit of fat, however, being not more than about 1/100,000 of the concentration found in the liver oils. Similar indications were also given by the lung and kidney oils of certain rats, but in general negative results were obtained in all organs other than the liver. A single test upon blood also gave a negative result.

5. Exposure of the rat to dietary vitamin A deficiency subsequent to carotene feeding led to no dramatic departure from the above distribution, except that after 68 days of such treatment unchanged pigment had virtually disappeared from the liver oil.

6. From the above evidence it is deduced that the conversion of carotene to vitamin A probably takes place in the liver, that the efficiency of the conversion is by no means quantitative and that the liver plays an important rôle in the regulation of the concentration of the vitamin throughout the remainder of the body.

My thanks are due to Dr L. J. Harris for his valuable criticism, and to Mr K. MacLennan of Lever Brothers, Ltd. for supplies of red palm oil. The care of the experimental animals was in the reliable hands of Mr A. Ward.

REFERENCES.

- Ahmad and Drummond (1930). *Biochem. J.* **24**, 27.
Buckley, Joss, Creech and Couch (1930). *J. Agric. Res.* **40**, 991.
Drummond and Baker (1929). *Biochem. J.* **23**, 275.
Kerppola (1930). *Skand. Arch. Physiol.* **56**, 181.
Moore (1929). *Lancet*, ii, 219.
— (1930). *Biochem. J.* **24**, 692.
Palmer and Kennedy (1920). *J. Biol. Chem.* **46**, 559.
Rosenheim and Webster (1927). *Biochem. J.* **21**, 111.
Rydbom (1930). *Biochem. Z.* **227**, 482.
Sherman and Boynton (1925). *J. Amer. Chem. Soc.* **47**, 1646.

XXXV. THE BIOCHEMISTRY OF ALUMINIUM.

II. EXCRETION AND ABSORPTION OF ALUMINIUM IN THE RAT.

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(Received January 7th, 1931.)

It has previously been shown by balance experiments with pigs that practically the whole of the aluminium provided as a supplement to the diet is voided in the faeces [Mackenzie, 1930]. With such animals there is a difficulty in devising a ration free from aluminium and accordingly this work has been supplemented by experiments with rats, where the ration could be built up by the use of highly purified foodstuffs.

The experiments of Flinn and Inouye [1928] on the excretion of aluminium by rats led them to believe that, when an aluminium salt was administered in the drinking water, 30 % of the aluminium was excreted in the urine and 70 % in the faeces. As details of the procedure are not given, it is uncertain whether contamination of urine by the faeces was prevented, and whether the drinking water was guarded against mechanical dissipation. The absorption of aluminium by rats, fed on diets containing aluminium over considerable periods, has been reported upon by Myers and Mull [1928]. There appeared to be some increase in the aluminium content of the tissues, though the method of analysis was not sensitive enough to give definite results. Wu [1929] states that rats fed on aluminised diets show an increased content of aluminium in the liver as compared with normal rats (0.97 mg. per 100 g. as compared with 0.59 mg. per 100 g.). References to experiments with other species were given in the previous publication [Mackenzie, 1930].

EXPERIMENTAL.

The chief difficulty in these experiments was to separate the three materials likely to contain aluminium, namely, the food, the urine and the faeces, as thoroughly as possible, while not interfering with the regular life of the animals. After a few trials the following method was adopted as the simplest and most satisfactory. The rats were kept in individual glass jars (15" long, 11" wide, 5" high) with a glass plate closing the mouth (fixed in position by wire), but leaving a gap of $\frac{1}{4}$ " at each side for air circulation. After preliminary cleaning the base of the jar was covered with a pad of filter-paper, made by folding a large sheet (40" \times 30") into two or three layers of suitable shape,

on which was superimposed a fine iron wire gauze mat mounted on a stout wire frame which fitted the bottom of the jar accurately. The food container was of special construction designed to prevent scattering of food; its dimensions and a section are given in Fig. 1. The lid was held in position by bayonet catches, and through the hole in its centre the rat could remove food by the mouth, but owing to the sloping guard ring was unable to drag it out with its paws. Water was supplied from drop-bottles, wired into position in the apertures at each side of the cage. The cages were cleaned out daily, the rat being removed to another cage; the faeces were brushed into a wide-mouth bottle and the gauze mat was taken out and shaken to remove any small particles of food, which were collected and returned to the food trough.

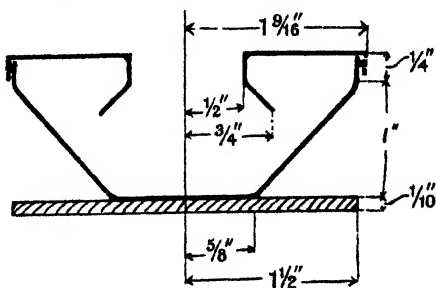


Fig. 1. Diagram of food trough.
Body of 12-gauge zinc sheet.
Base of hard lead sheet.

The rats used in these experiments were members of larger groups of 30–40 animals which were employed on feeding and fertility tests; these groups of rats were chosen from a number of young albino or Norwegian rats recently weaned, which were divided into two symmetrical groups, one receiving a synthetic diet approximately free from aluminium and adequate for growth, the other a similar diet with the addition of 0.1 % aluminium. Growth was usually regular, the animals gaining in weight steadily from 40 g. to 200 g. in the course of 12–16 weeks. In the first two metabolic experiments, fully-grown animals were used, three animals on the control, and three on the aluminised diet, and each animal occupied a separate cage. In the third experiment, animals 12 weeks old, averaging 120 g. in weight were employed, and the three animals of each group were placed in the same cage. It was hoped by this means to reduce the amount of aluminium which could not be accounted for, but the improvement in the result was not noticeable.

Analysis of material.

The food. Sufficient amounts of the control diet and aluminised diet were removed from the main bulk of the stock rations, well mixed, and sampled. The control diet was analysed by the spectrographic method (a modified version of the method used by McCollum, Rask and Becker [1928]) and the aluminised diet by the method of Schmidt and Hoagland [1912].

The faeces. These were collected daily during the metabolic period and at its conclusion were dried, ground and analysed, the control group by the colorimetric method [Myers and Morrison, 1928], the aluminised group by the gravimetric method.

The urine. The filter papers were removed from the cages, dried, brushed

lightly to remove any traces of adherent food, ashed, and analysed by the colorimetric method. Blank determinations were made on a few fresh filter papers.

Table I. *Diets.*

	Exps. 1 and 2		Exp. 3	
	Control	Aluminised	Control	Aluminised
Yeast	25 parts	25 parts	24 parts	24 parts
Caseinogen	90 "	90 "	88 "	88 "
Salt mixture 185	20 "	20 "	20 "	20 "
Cellulose	10 "	10 "	10 "	10 "
Butter	140 "	140 "	80 "	80 "
Starch	215 "	195 "	215 "	200 "
A.P.B.P.*	—	20 "	—	15 "
Al. content mg./100 g.	0.11	106.5	0.11	85.4

* This represents a commercial brand of baking powder, containing acid calcium phosphate and sodium aluminium sulphate; it was decomposed with water and dried, the product containing about 3 % of aluminium.

Table II. *Results of experiments carried out on groups of three rats.*

	Exp. 1		Exp. 2		Exp. 3	
	Control	Aluminium	Control	Aluminium	Control	Aluminium
Food eaten (g.)	540	540	362	393	356	321
Duration (days)	12	12	7	7	9	9
Food eaten per rat daily	15	15	17.2	18.7	13.2	11.9
Total aluminium ingested (mg.)	0.59	575.0	0.395	418.5	0.39	274.1
Aluminium output (mg.):						
(a) in urine	0.006*	0.026*	0.006*	0.014*	0.008	0.024
(b) in faeces	0.47	484.2	0.29	394.1	0.44	261.3
Total aluminium output (mg.)	0.476	484.2	0.296	394.1	0.448	261.3
Output as % ingestion	80.7	84.2	74.9	94.2	114.9	95.4

* Total of three determinations.

Urinary excretion. In arriving at the figure for urinary excretion it is necessary to take into account the value of the blank as determined on several fresh sheets of filter-paper. The average of six blanks was 0.003 mg., and the largest single figure obtained was 0.008 mg. Since the analytical method employed is of doubtful accuracy with amounts of aluminium less than 0.005 mg., only two of the results for urinary excretion can be accepted as definitely indicating the presence of aluminium—the "aluminium groups" of Exps. 1 and 3. In these cases, it is possible that a small amount of the food employed in the experiment may have passed through the gauze mat and become incorporated in the paper when analysed; an amount of 20 mg. so included would be sufficient to account for the amount of aluminium observed. But even if this is not so, the amount of aluminium is so small in relation to the total ingestion as to make it of little significance.

The faeces. (a) *Recovery of aluminium ingested by control animals.* The disparity in the figures of intake and output is probably due to analytical error. The amount of aluminium is in all cases very small, and the systematic error

is consequently much greater than usual. Combination of all the results for the control animals gives an average recovery of 90.2 %, or an average possible absorption of 0.04 mg. per rat.

(b) *Recovery of aluminium ingested by aluminium-fed animals.* In the second and third experiments a high level of recovery, approximately 95 %, was obtained, but the recovery in the first experiment was unexpectedly low. This was assumed to be due to coprophagy, though this had not been observed during the experiment; it may have occurred, since the rats were supplied with a fixed amount of food daily, and it is possible that this was not sufficient in the case of one or two rats. In the subsequent experiments, an excess of food was always supplied, and only the total weight eaten for the whole period was ascertained. The recovery of aluminium was approximately equal in the two latter cases, and it seems doubtful whether attention to detail could reduce the unavoidable loss much further. Two causes may operate to cause error: mechanical losses, and variation in the rate at which the animals consume their food. It seems unlikely that the latter can be a serious source of error. The use of three animals over a period of 7 days would reduce such variation to a very small percentage of the total.

*Possible absorption of aluminium as determined by analyses
of the animal organs.*

In the course of the feeding and rearing experiments with which the above metabolic experiments were connected, a number of the adult rats were killed and their internal organs were analysed for aluminium. The results were considered applicable to the problem immediately under consideration, as these rats received exactly the same food and treatment throughout their life-period. The analyses were conducted on the organs removed from 8–10 rats, so as to minimise systematic errors, experience having shown that with the smaller amount obtained from 3 rats these were apt to be unduly large.

The analytical data for two groups of rats are given in Table III. Series A contained the rats used in Exps. 1 and 2. Series B contained the animals used in Exp. 3. All rats were full-grown and healthy, with an average weight in excess of 200 g.

Table III.

	No. of rats examined	Aluminium found in mg./100 g. fresh material				
		Liver	Lung	Heart	Stomach	Small intestine
Series A:						
Control diet	10	1.8	0.32	—	3.8	2.8
Aluminium diet	8	2.2	0.22	—	1.1	2.9
Series B:						
Control diet	9	1.6	0.16	0.20	1.35	1.15
Aluminium diet	8	1.2	0.14	0.24	2.6	2.2

These results cannot be considered as evidence that absorption of aluminium occurs through ingestion of aluminised food; the variations between the

different groups are not greater than have been obtained in other series of analyses in which no aluminium was fed. The general trend of these experiments is to show that aluminium plays no important part in the metabolism of the rat, and supports the theory that aluminium, when ingested, is quantitatively excreted through the alimentary tract.

SUMMARY.

Rats receiving a diet containing aluminium excrete the aluminium entirely by way of the alimentary tract, and there is no clear evidence that any of the aluminium is excreted in the urine.

No definite evidence of absorption of aluminium by the internal organs of such rats has been established.

REFERENCES.

- Flinn and Inouye (1928). *J. Amer. Med. Assoc.* **90**, 1010.
Mackenzie (1930). *Biochem. J.* **24**, 1433.
McCollum, Rask and Becker (1928). *J. Biol. Chem.* **77**, 753.
Myers and Morrison (1928). *J. Biol. Chem.* **78**, 615.
—— and Mull (1928). *J. Biol. Chem.* **78**, 605.
Schmidt and Hoagland (1912). *J. Biol. Chem.* **11**, 387.
Wu (1929). Abstracts of Master's theses, Ohio University, **2**, 45.

XXXVI. CALCIFICATION OF THE BONES OF RATS ON A DIET LOW IN ERGOSTEROL.

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(Received January 7th, 1931.)

THE statement is frequently made that ergosterol is a substance which cannot be made in the animal body but there does not seem to be any real evidence that this is the case.

Beumer [1927] attempted to trace the fate of ergosterol ingested by an infant; of 0.4 g. ingested, he only succeeded in recovering 0.147 g. from the stools, but the observation does not shed much light on the problem.

In contrast with earlier workers both Channon [1925] and Randles and Knudson [1925] concluded that the rat can synthesise cholesterol. One of the chief difficulties in such an experiment is to supply the fat-soluble vitamins to the experimental animals free from sterols. Channon met this difficulty by using the unsaponifiable matter from cod-liver oil, after having first freed it from cholesterol by precipitation with digitonin; such a procedure seems satisfactory and would presumably leave the rat supplied with vitamins A and D. Randles and Knudson used dried alfalfa leaves, which had been extracted with cold ether; the method was held to remove the sterols from the leaves, while not extracting the vitamin A; the extraction of the sterols by this method could however hardly be regarded as complete and the experiment would seem to lack conclusiveness on this ground.

Even if cholesterol can be synthesised in the rat's body, as Channon's work indicates, it cannot therefore be concluded that this is also true of ergosterol. It seemed possible that the problem might be attacked indirectly, not by an attempt to estimate the ergosterol balance but by ascertaining whether the administration of diets, rich in ergosterol on the one hand, and rendered as deficient as possible in it on the other, had any influence on calcification in rats irradiated with ultra-violet light and deprived of any source of vitamin D through the mouth.

It was obvious that the experiment must be a long one, in order to aim at a depletion of any reserves of ergosterol in the rat; the diet would therefore have to be as complete as possible, so that prolonged well-being might be assured, while it must at the same time be rendered as free as possible from vitamin D and from sterols. The chief problem lay in the preparation of sources of vitamins A and B which should fulfil this requirement.

The experiment lasted about 4 months and was therefore a prolonged observation on the behaviour of rats on a fat-free diet. Certain observations were made in the course of it, on the occurrence of a condition of scaliness of the tail, described in particular by Burr and Burr [1929], as occurring on diets free from fat. These observations are included in an ensuing note.

EXPERIMENTAL.

Preparation of the diet. The diet was composed as follows:

Caseinogen	...	20
Wheat starch	...	65
Salt mixture	...	5 (McCollum 185 [McCollum and Davis, 1914] ¹)

Daily supplements were given separately of a vitamin A concentrate equivalent to 1.0 g. of the original spinach, and of a vitamin B concentrate equivalent to 1.0 g. of dried yeast.

The caseinogen used was that of the British Drug Houses, "fat- and vitamin-free," which is extracted with alcohol and ether.

The wheat starch received one extraction with cold light petroleum, by which means a small amount of a yellow oil was removed.

The caseinogen, starch and salt mixture were mixed with freshly distilled water and steamed in a double saucepan. By this means the starch grains were burst and any risk of refection obviated [see Roscoe, 1927, 1]. The mechanism of refection is not understood, but if, as seems possible, it depends on the elaboration of the B vitamins by some organism within the intestinal tract and their subsequent absorption by the rat, it would also appear possible that such organisms, particularly if they were yeasts, might also elaborate ergosterol and provide it to the rat. It was advisable therefore to take special precautions against refection.

An extract from spinach leaves was prepared to be a source of vitamin A. It was hoped that this when rendered sterol-free would supply a source of vitamin A which would also be free from vitamin D. Willimott and Wokes [1927] found that an ether extract of spinach, when fed in the equivalent of 5.0 g. of fresh spinach daily, supplied adequate vitamin A and had no significant influence on calcification, but Chick and Roscoe [1926] and Roscoe [1927, 2] found that the fresh leaves of summer spinach did exercise a small but definite antirachitic effect. It is certainly possible to render spinach strongly antirachitic by artificial ultra-violet irradiation, but the amount of vitamin D appears to be negligible unless the spinach is exposed to special

¹ Composition of salt mixture:

Sodium chloride	51.9
Magnesium sulphate	164.0
Sodium dihydrogen phosphate	104.1
Dipotassium hydrogen phosphate	286.2
Calcium phosphate	162.0
Calcium lactate	390.0
Ferric citrate	35.4

conditions of irradiation or insolation. At any rate the ether extract of a green leaf appeared to offer the best hope of providing a source of vitamin A, devoid of vitamin D. In the present state of knowledge, it is easy to see that it would have been better to have used recrystallised carotene as the source of vitamin A, since Moore [1929] has shown that purified carotene has no antirachitic activity. At the time, however, when the present experiment was carried out, it seemed unwise to proceed further in fractionation than to prepare a light petroleum extract of spinach, to saponify it and to use what was left of the unsaponifiable fraction after the sterols had been precipitated with digitonin.

Preparation of the extract of vitamin A. Two batches of extract were prepared. One was derived from 1500 g. of fresh prickly-seeded spinach (*Spinacia oleracea*) gathered in May. The product was used for a preliminary test to establish the vitamin A value of the extract. The second batch of 4200 g. of similar material was gathered at the end of September and beginning of October and the product was used for the main experiment.

The leaves were sorted and the larger pieces of stalk were removed. The leaves were dipped in boiling water and dried in front of a fan at a temperature of 37°. The dried leaves were powdered and sieved and extracted repeatedly with light petroleum (B.P. 40–60°), the solvent being subsequently distilled off at about 50°. From 1500 g. of fresh spinach about 2.6 g. of solid extract were obtained and from 4200 g. about 9.6 g. The extract was taken up with ether.

Saponification was carried out with sodium ethoxide, freshly prepared from metallic sodium and ethyl alcohol. Part of the alcohol was evaporated and water was added to the remaining solution, which was repeatedly shaken with fresh amounts of ether until the ether fraction ceased to show more than a little coloration. The ether was distilled off from the collected extracts, and after drying the yield of unsaponifiable matter was, from 1500 g. of spinach, 1.95 g., and from 4200 g., 3.8 g.

The unsaponifiable fraction was treated with digitonin in alcoholic solution. The solution was filtered to free it from the insoluble digitonide, which was washed with ether and alcohol. The alcohol was taken off and the residue was then taken up with ether and filtered to free it from the ether-insoluble excess of digitonin. When the ether was finally removed, a deep orange-coloured fraction was left, amounting to 0.92 g. from 1500 g. of spinach and 3.06 g. from 4200 g. of spinach.

The first batch of material, derived from 1500 g. of fresh spinach was taken up in hardened cottonseed oil and was fed to rats after a depletion period on a diet deficient in vitamin A. An amount corresponding with 1.0 g. of fresh spinach daily was found to supply a sufficient source of vitamin A over an extended experimental period of 70 days. The writers have found 0.02 and 0.03 g. of fresh spinach to supply adequate vitamin A for maintenance in the rat for an experimental period of 35 days [Hume and Smith, 1930].

The second batch of material, derived from 4200 g. of fresh spinach, was used for the sterol-free experiment. For this purpose the material was taken up with liquid paraffin (Internol of Messrs Allen and Hanbury); the preparation was stored in a brown bottle at about 0°; in these circumstances the yellow colour was well maintained and the preparation retained potency over a long period. It was so made up that one drop of the liquid paraffin contained the equivalent of 1.0 g. of fresh spinach; each rat received one drop daily of the preparation throughout the whole period of the experiment.

Preparation of the extract of the B vitamins. The concentrate of B vitamins was prepared from brewer's yeast by the method described by Chick and Roscoe [1929].

The yeast was washed four or five times with ice-cold water and pressed. About 15 kg. of such moist yeast were thrown into about 30 litres of boiling distilled water containing 0.01 % of acetic acid. The whole was again brought to the boil and filtered through Büchner funnels while hot. The clear filtrate was concentrated to a convenient small bulk, acidulated with sulphuric acid to about p_H 3.0 and stored at 0°. It was diluted and filtered again just before use.

The preparation was tested on rats, kept on open wire screens and fed on a diet deficient in the B vitamins, *i.e.* caseinogen (British Drug Houses, "fat- and vitamin-free") 300, wheat starch 750, hardened cottonseed oil 225, salt mixture (McCollum 185) 75, cod-liver oil 3–5 drops per rat daily. The diet was mixed with water and steamed. On an equivalent of about 1.0 g. of dried yeast daily, young rats grew well for an experimental period of 35 days. A similar dose was used throughout the main experiment. No source of vitamin C was given.

For a part of the animals ergosterol was added to the diet and for that purpose ergosterol supplied by the British Drug Houses was used. It was dissolved in "Internol" so that one drop contained $\frac{1}{10}$ mg. In this concentration it showed a tendency to crystallise out so that the bottle had always to be shaken before use.

Method of experiment.

The rats used were of the Lister Institute black and white strain; they were about 33–39 g. in weight and about 20–23 days old. They were kept singly in cages on open wire grids of mesh 3 squares to the inch to prevent consumption of faeces. The cages were washed several times a week, if necessary; it was feared that even a small contamination with faeces might supply ergosterol to the rats, through micro-organisms in the faeces.

Four litters of rats were used and were distributed evenly as regards sex and litter amongst four experimental groups; each of the four groups included five individuals, which were identical in sex and litter for each group. For the 1st week of experiment all four groups were treated alike; after that two of the groups received a daily addition of one drop of liquid paraffin, containing 0.01 mg. of ergosterol, while the other two groups received the same amount of liquid paraffin only. The experiment lasted 120 days, except in the case of one animal in each group (one litter), where it lasted 112 days.

About the 80th day of experiment it was hoped that the rats' ergosterol reserve, in those groups not receiving ergosterol, might be exhausted. Irradiation with a mercury vapour quartz lamp (Hewittic Electric Co.) was therefore instituted for one of the two groups receiving ergosterol and for one of the two not receiving ergosterol. The four groups therefore were receiving treatment as follows:

Group 1. No irradiation	No ergosterol
Group 2. No irradiation	Ergosterol
Group 3. Irradiation	No ergosterol
Group 4. Irradiation	Ergosterol

Irradiation was for 10 minutes every weekday at a distance of about 60 cm. It was found necessary to shield the eyes of the rats from time to time,

whenever they became sore from the irradiation, otherwise the animals ceased to thrive.

The animals were weighed at regular intervals. At the end of the experiment, all were killed and the femur and tibia of both legs were removed. These were dried, extracted with ether and alcohol and the ash content was determined.

RESULTS.

The rats did well throughout the experiment and all survived the full period. The eyes of those which had not been irradiated were all normal or nearly normal at the end of the experiment; the condition of the eyes in those which had been irradiated could not be judged as regards xerosis, since, in spite of being frequently shielded, the eyes of these rats were rendered abnormal by the action of the mercury vapour lamp. Only three out of the twenty animals developed "snuffles" and only two showed small patches of congestion in the lungs at autopsy. The vitamin A supply throughout the experiment would therefore appear to have been quite adequate.

All the four rats belonging to one litter (No. 1228) developed small sore patches on the skin about the face and shoulders, towards the close of the experiment. This condition has often been observed before by the writers, in rats on other diets; it appears to be caused by an abnormal multiplication of lice, which cause irritation to the rat; the writers are inclined to correlate it with an unknown dietary deficiency.

All the rats developed some signs of the "scaly tail" condition, described by Burr and Burr [1929]. The tails were excoriated, shiny, annulated and scaly in parts and in some instances there were signs of the same condition on the skin of the feet. It would be natural to conclude with Burr and Burr that the condition was due to the absence of fat from the dietary, were it not that the same condition developed in the rats which were used for testing the material employed as a source of vitamin B. These latter rats received about 16 % of hardened cottonseed oil in their diet, together with 3-5 drops of cod-liver oil per head daily. The presence or absence of fat could not therefore have been the factor governing the development of scaly tail. The factors differentiating these experiments from others which the writers have carried out, and in which the condition has not been seen, would appear to be the type of caseinogen, the source of the B vitamins and the type of cage, with open wire screens to prevent coprophagy. A further investigation and discussion of the problem is included in the following paper.

Growth was strong at the start, being at first fully normal but after about 50 days, in most cases, it tended to slacken off; females had then reached a weight of about 150 g. and males of 200 g. An increase in the supply of B vitamins towards the close of the experiment did little to restore the growth rate and it is probable that the slackening was due to the lack of an unknown dietary factor, either that one, the lack of which caused the scaly tail condition, or another. A little acceleration of the growth rate took place after

the institution of irradiation about the 80th day of experiment in Groups 3 and 4, but if growth was being limited in any case by the lack of some other factor, any benefit to growth from the administration of vitamin D would be thereby obscured. The total growth response in the four groups, in the period after the institution of irradiation in Groups 3 and 4, was as follows:

Group 1. No irradiation, no ergosterol	106 g.
Group 2. No irradiation, ergosterol	112 g.
Group 3. Irradiation, no ergosterol	129 g.
Group 4. Irradiation, ergosterol	147 g.

The figures appear to show a small but distinct superiority in growth in the two groups which were irradiated.

Table I. *Percentage ash in the dried extracted bones of rats fed on a diet made as deficient as possible in ergosterol (Group 1), to which ergosterol was added (Group 2), which were irradiated with ultra-violet light for the latter part of the experiment (Group 3) and which were both irradiated and received added ergosterol (Group 4).*

Litter No.	Sex	No irradiation and no ergosterol	No irradiation and ergosterol	Irradiation and no ergosterol	Irradiation and ergosterol
1228		60.6	62.4	61.9	62.4
1227	♂	59.0	59.2	60.7	60.4
1255	♂	60.3	61.0	61.9	61.8
1255	♀	60.8	61.7	62.3	61.7
1272	♀	59.8	61.2	61.5	61.0
Average		60.1	61.1	61.7	61.5

The percentage ash in the dried extracted bones is set out in Table I. It is seen at once that all bones, even those in the negative control, Group 1, without ergosterol or irradiation, show a very high measure of calcification. In comparison with these, however, the two irradiated groups show a small but definite superiority in calcification; the range in each group is so small and the superiority of each individual irradiated rat over its corresponding litter mate in the negative control group is so regular that it would appear permissible to accept the result, in spite of the relatively high calcification in all groups. There is no difference between the calcifications of the two irradiated groups, indicating that abundance or deficiency of ergosterol in the diet made no difference to the calcification when the rats were exposed to ultra-violet light from a powerful artificial source. The second group, those rats which received ergosterol without irradiation, show a curious result. The individual values are more scattered and, while on the whole, barely inferior to the values in the irradiated groups, are definitely superior to those in the negative control group. The result seems to suggest that a rich supply of ergosterol in the diet promotes calcification when the only source of ultra-violet light is diffuse daylight in a North room. It must not be forgotten that ergosterol feeding in Groups 2 and 4 took place over the whole period of the experiment.

The whole experiment is marred by the high calcification of the negative controls, which is not easy of explanation. The salt mixture used is certainly not one on which a low calcification would be expected, but past experience suggests that some other explanation is needed. The reserves of the rats in vitamin D might have been high, but this has not been found to be the case when similar rats from the Lister Institute stock have been fed on rickets-producing diets. The only other explanation would seem to be that the vitamin A concentrate, prepared from spinach, also contained vitamin D; that possibility has already been discussed and dismissed as improbable, particularly in view of the smallness of the dose used, which was the equivalent of 1.0 g. of fresh spinach.

The result suggests that limitation of ergosterol in the diet did not act as a limiting factor in calcification when the rats were powerfully irradiated with ultra-violet light. It is not however possible to deduce from this result whether ergosterol can be synthesised in the rat's body or not; that some measure of synthesis takes place is one possible interpretation, but ignorance as to the rat's possible reserve of ergosterol and uncertainty as to whether the diet was completely devoid of, or only relatively deficient in, ergosterol, make it impossible to draw a definite conclusion, the results being such as they are.

If synthesis did take place, addition of ergosterol to the diet was still able to raise the calcification to a slightly higher level, when the supply of ultra-violet light was only very small.

SUMMARY.

1. Experiments are described in which rats were fed on a diet made as free as possible from ergosterol, with the object of ascertaining to what extent this sterol can be manufactured in the animal body. Other rats were fed on the same diet with an abundant addition of ergosterol. After about 80 days half the rats in each series were irradiated with a mercury vapour quartz lamp daily, for about 40 days. At the end of the experiment the percentage ash in the dried fat-free bones of all the rats was determined.

2. The bones of all the rats showed a high percentage of ash. That in the bones of the irradiated rats receiving ergosterol was the same as in those of the irradiated rats not receiving ergosterol. The ash of the group receiving neither irradiation nor ergosterol was significantly lower than that of the other groups. The group which received ergosterol but not irradiation showed an ash content of the bones which did not differ significantly from that of the two irradiated groups.

3. No answer therefore was obtained to the main question of the enquiry, *i.e.* the possibility of ergosterol synthesis in the rat body. Since however, when the supply of ultra-violet light was abundant, a variation in the amount of ergosterol in the diet made no difference to the calcification, it must be concluded that the rat was, in those circumstances, sufficiently supplied with ergosterol. Such ergosterol may have been supplied by some measure of

synthesis or it may have been derived from a residual amount in the diet or from the faeces, for in spite of all precautions it was found impossible to prevent the rat from consuming traces of the latter.

4. When, however, the supply of ultra-violet light was very small, the supply of ergosterol from any of the possible sources just mentioned was not sufficient to promote the maximum calcification, which was only then attained when abundant ergosterol was added to the diet.

5. In the course of these experiments, which represent a prolonged maintenance of rats on a fat-free diet, the animals developed the "scaly tail" condition of Burr and Burr, who attributed it to lack of fat in the diet. The observation is discussed in the next paper.

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REFERENCES.

- Beumer (1927). *Klin. Woch.* **6**, 2, 941.
Burr and Burr (1929). *J. Biol. Chem.* **82**, 345.
Channon (1925). *Biochem. J.* **19**, 424.
Chick and Roscoe (1926). *Biochem. J.* **20**, 137.
—— — (1929). *Biochem. J.* **23**, 504.
Hume and Smith (1930). *Lancet*, ii, 1362.
McCollum and Davis (1914). *J. Biol. Chem.* **19**, 245.
Moore (1929). *Biochem. J.* **23**, 1270.
Randles and Knudson (1925). *J. Biol. Chem.* **66**, 459.
Roscoe (1927, 1). *J. Hyg.* **27**, 103.
—— (1927, 2). *Biochem. J.* **21**, 211.
Willimott and Wokes (1927). *Biochem. J.* **21**, 887.

XXXVII. THE RELATION OF A FAT-FREE DIET TO THE SCALY TAIL CONDITION IN RATS DESCRIBED BY BURR AND BURR.

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EXPERIMENTS made before the discovery of the fat-soluble vitamins with the object of determining the indispensability or otherwise of fat in the diet have little value. No effort was made to supply the fat-soluble vitamins and failures which were really due to a deficiency of them were attributed to the lack of fat as fat.

As early as 1918 Hindhede [1918] concluded from prolonged dietary experiments on young men that fat was not necessary if a daily ration of fruit and vegetables, sufficient to supply the fat-soluble vitamins, was consumed.

Animal experiments in which the distinction was drawn between fat as fat and fat as the vehicle for the fat-soluble vitamins were first made by Osborne and Mendel [1920-21] and by Drummond and Coward [1921]. In the observations of Osborne and Mendel the diets were not completely devoid of fat, they were only relatively very low and fat-soluble vitamins were supplied as dried alfalfa. The rats thrived and grew well and the authors concluded that "if true fats are essential for nutrition during growth the minimum necessary must be exceedingly small." In Drummond and Coward's experiment the fat in the diet was reduced to a trace and fat-soluble vitamins were supplied as the unsaponifiable fraction of fish-liver oil. The rats grew well on the diet and reproduced, and the conclusion of Hindhede and of Osborne and Mendel was completely borne out, that fats, while being a convenient form of energy-bearing food, are only indispensable as carriers of the fat-soluble vitamins; when a supply of these is otherwise assured, fats can be omitted from the dietary.

For some years this view remained unchallenged, until Evans and Burr [1928] and Burr and Burr [1929] announced a new dietary deficiency in fat-free diets, which was relieved by the administration of fatty acids. The rats on the fat-deficient diet grew sub-normally and developed a scaliness and annulation of the tail, which might become necrosed, so that 1-3 cm. of the tip might be lost; the skin of the feet and also of the whole body ultimately became affected and the animals finally declined and died.

McAmis, Anderson and Mendel [1929] describe some experiments in which

rats on fat-free diets grew and thrived less well than those on diets containing fat, though the fat-soluble vitamins were supplied as the unsaponifiable fraction of cod-liver oil. One case is described where the tail of the rat became dark and gangrenous, but a condition as severe as that described by Burr and Burr was not observed.

The condition of "scaly tail" which occurred among the rats used by the present writers in the experiment described in the preceding paper [1931] appears to be identical with that described by Burr and Burr, but as has already been stated, it could not be referred to the lack of fat in the dietary, since it occurred also among rats receiving abundant fat. The factors to which it might be attributed appeared to be rather the type of caseinogen, the source of B vitamins and the type of cage used, with open wire grids to prevent coprophagy. The condition has also been observed in the Lister Institute by Chick, by Roscoe and by Aykroyd (personal communications) on a variety of diets but always when using cages with coarse wire flooring, on diets containing specially purified caseinogen and usually with yeast derivatives and yeast fractions, rather than with whole yeast as the source of B vitamins. Experience in this Institute is not incompatible with the view that modification of the supply and source of yeast vitamins, under which heading the use of anti-coprophagy cages is included, is the responsible factor in the production of scaly tail.

Analysis of the experimental results of the other workers already cited is not inconsistent with such an interpretation. The early experiments of Drummond and Coward and of Osborne and Mendel, in which the rats thrived on a fat-free diet, would probably have been carried out in cages with solid bottoms as was usual at that date. Evans and Burr made a first series of experiments in which cages with solid bottoms were used, while in all the subsequent ones the rats were housed on wire grids. It is remarkable that while in each experiment rats receiving fat grew better than those which did not, yet those which received fat and were caged on wire grids grew less well than ones which did not receive fat, but were housed in cages with solid bottoms. Less pure caseinogen seems also to have been used in the experiment where the cages had solid bottoms. The B vitamins were supplied as 0.7 g. daily of Fleischman yeast. No symptoms are described as having occurred in this experiment.

In the experiment of McAmis, Anderson and Mendel the conditions were somewhat similar; the experiments were carried out using, partly, cages "consisting largely of glass" and, partly, cages with floors of open wire mesh. Harris yeast concentrate was used as a source of the B vitamins and later it was supplemented with 400 mg. of yeast daily. Inferior growth was shown by the rats on the fat-free diet and in one instance a tail lesion was observed, but the experiments are not well described and it is clear from the account of the autopsies that the rats on the fat-free diet, which received their fat-soluble vitamins as unsaponifiable fraction of cod-liver oil, were partly de-

ficient in vitamin A. It is not possible to obtain much evidence from an analysis of these experiments.

In the work of Burr and Burr the rats were housed throughout on coarse wire grids (two meshes to the inch); they received a diet which contained no fat, specially purified caseinogen and ether-extracted yeast. In these conditions the symptoms already described developed severely and could be cured by 10 drops of lard or 1.0 g. of liver daily, but not by 0.7 g. of whole yeast daily. The conditions here were more drastic and the syndrome developed fully, but the cure with 10 drops of lard and the failure with 0.7 g. whole yeast do not appear to support a theory of some deficiency in the yeast vitamin complex. It might however be that in the drastic conditions of the experiment 0.7 g. of dried yeast did not supply enough of the factor, after the condition had developed severely. Aykroyd (personal communication) also only secured slow improvement of the condition when established to a severe degree, with 1.0 g. of dried yeast but a cure was brought about with a daily dose of 1.0 g. dried liver. The failure with 0.7 g. of dried yeast definitely puts any question of a deficiency of vitamin B₂ out of court.

Some work of Evans and Lepkovsky [1929] however suggests in a most interesting manner that the curative action of fat for scaly tail may be exercised by making good some deficiency in the yeast vitamin complex. They show that fat in the diet can have the effect of reducing the amount of B vitamin which the rat needs and conclude that the more fat there is in the diet up to a point the less antineuritic vitamin B₁ is needed. They do not consider that this sparing action of fat is extended to vitamin B₂, but a careful examination of their charts suggests that it is, though less effectively than in the case of vitamin B₁. If fat, therefore, can have a sparing action on the B vitamin complex, an explanation is offered of Burr and Burr's observations, which is also compatible with an ascription of the deficiency which produces scaly tail to lack of some factor within the yeast vitamin complex. Evans and Lepkovsky go no further than conjecture with regard to the mechanism by which the sparing action is produced; they suggest possible alterations in the rat's intestinal flora. The present writers would tentatively suggest, though they have no evidence in support of their suggestion, that the addition of certain fats might operate by altering the consistency of the faeces, rendering them more pasty and less liable to fall through the wire grids and thereby promoting coprophagy. At any rate an examination of the literature and a comparison of the results with the writers' own experience made it seem worth while to test further the hypothesis that the aetiology of the scaly tail syndrome lies in variation of the conditions governing the supply of the yeast vitamin complex.

EXPERIMENTAL.

The object aimed at was to compare the condition of rats which received diets containing fat or no fat, and were kept on wire screens and on solid bottoms respectively.

It was decided to use the commercial yeast extract marmite as the source of B vitamins.

Twelve rats were used which were derived from four different litters, so that it was not possible to balance four experimental groups perfectly in this respect.

For the first 50–60 days of experiment all the rats received the following diet:

Caseinogen (British Drug Houses, fat- and vitamin-free)	...	20
Rice starch	72
Marmite yeast extract	4
Salt mixture (McCollum 185)	4

Vitamin D was given separately as 0.004 mg. of irradiated ergosterol in ethyl oleate daily, but no vitamin A was given in this period of the experiment as the animals were originally intended for another purpose.

The rats were kept in cages with a floor of such finely perforated zinc that, so far as coprophagy was concerned, it amounted to a solid floor.

About the 50th–60th day the animals showed signs of failing from lack of vitamin B₁; marmite was therefore increased to 15 % of the diet. At the same time fat was introduced into the diet of six rats as 10 % of hardened arachis oil in the place of 10 % of starch, while the remaining six rats remained on the same diet devoid of fat. Of the six rats receiving fat in the diet, three were placed in cages with coarse screens (9 to the square inch) and three remained on the same floors as before: the six on a diet without fat were similarly divided. The irradiated ergosterol from this point also was administered in solution in liquid paraffin instead of in ethyl oleate. Experiments with carotene had shown how quickly it is oxidised in ethyl oleate solution, and it was feared that vitamin D might suffer oxidation in the same way.

For the next 40 days, for the purpose of another observation, the six rats on fine screens received their vitamin A as 0.006 mg. of carotene in liquid paraffin daily, while those on coarse wires received it as 0.04 g. of fresh green cabbage daily. At the end of the 40 days, *i.e.* about the 100th day of experiment, all the rats received their vitamin A as cabbage. At the same time, since any symptoms of scaly tail so far shown were very slight, the marmite was reduced once more to 8 %; it was deemed that the supply of vitamins B₁ and B₂ would be again endangered if it were reduced any lower.

About the 180th day as the rats on the fine wires showed very little sign of the disease coarse screens were substituted.

About the 200th day, when it was clear that fat in the diet was making no difference, vitamins A and D were administered more conveniently as 5 drops of cod-liver oil daily, instead of as cabbage and irradiated ergosterol.

The total duration of the experiment was about 290 days.

On the regime above described, no rat developed a severe condition of scaly tail but all showed a recognisable degree of it, except one which died

on the 126th day. At autopsy it was found to have a cystitis, with stones in the bladder; the vitamin A supply was therefore probably insufficient for it. The growth of the animals is not considered in the results; the supply of vitamins B₁ and B₂ was insufficient for good growth over the major part of the experiment, so that the growth performances have no significance. The behaviour of the groups was as follows.

Group 1. Diet + fat. Coarse screens. Symptoms developed early and comparatively severely, with a well-marked black necrotic tip to the tail in one case, moderately in the second case and slightly in the third. The most severe case showed slow improvement when treated with 0.2 g. dried yeast for 72 days and became cured, so that even the black tip to the tail disappeared, when treated with 0.4 g. daily for 41 days. The second case improved very slowly on 0.1 and 0.2 g. daily of dried yeast, but was almost cured when the experiment was ended after it had been for 21 days on 0.4 g. The third showed little improvement when treated with 0.2 and 0.4 g. of autoclaved (120° for 5 hours) dried yeast daily but was improved after 27 days on 0.4 g. of dried unheated yeast.

Group 2. Diet - fat. Coarse screens. All the three rats developed well-marked scaliness and two showed very small necrotic black tips to their tails, one of which made its appearance while the rat was under treatment with autoclaved yeast. Treatment with 0.4 or 0.5 g. of autoclaved yeast brought about no improvement, but when the dose was changed to one of 0.4 g. of yeast, not autoclaved, over a period of 20 days, two of the rats showed improvement and one was cured.

Group 3. Diet + fat. Fine screens. Symptoms developed very slightly while the rats were on the fine screens but increased after coarse ones were substituted. One rat died without symptoms before that date was reached. The two survivors were treated with 0.2 and 0.5 g. autoclaved yeast with no benefit; a black tip to the tail was developed in one case after the treatment had begun. Improvement took place when the rats were treated with 0.4 g. dried yeast for 12 and 27 days respectively.

Group 4. Diet - fat. Fine screens. The tail of one rat remained normal up to the removal of the fine screens; the other two showed slight but slowly increasing symptoms. After the substitution of coarse screens the degree of scaliness increased and two rats developed black tips to their tails, one of them whilst under treatment with autoclaved yeast. No improvement took place with doses of 0.2, 0.4 or 0.5 g. of autoclaved yeast. With a daily dose of 0.4 g. of dried yeast improvement took place after 10, 17 and 18 days respectively.

DISCUSSION.

The condition was not developed severely enough to give very sharp results. On 15 % of marmite in the diet, it scarcely developed at all; it was only after the marmite was reduced again to 8 % that the condition became definite. Symptoms occurred approximately equally in the groups with and without

fat, though they were perhaps slightly more severe in the groups without fat. Four of the six rats without fat developed black tips to their tails, while only two did so in the two groups which received fat. On the other hand the most severe case, with the largest black necrotic area on the tail, was that of a rat which received fat in the diet.

The symptoms developed slightly or not at all when the rats were housed on fine screens and became more marked when coarse ones were substituted.

The condition was unaffected or even grew worse while the animals were being treated with 0.2–0.5 g. daily of autoclaved yeast. Dried yeast in a daily dose of 0.4 g. produced improvement, and the most severe case, on which treatment was begun early, was completely cured.

The caseinogen and the coarse wire grids were the same as were used in the sterol experiment, where the symptoms were more marked; the source of yeast vitamins was however different and one is inclined to suggest that in marmite the vitamin factors are not sufficiently unbalanced for the production of scaly tail. Even 0.4 g. of yeast only slowly cured the condition when it was very mild; it is easy to imagine that considerably larger amounts might fail to cure a very much more drastic deficiency.

The results of this experiment lend some support to the hypothesis that the production of the scaly tail condition is influenced by factors which influence the supply of the yeast vitamin complex. In certain circumstances the presence or absence of fat in the diet appears to be one of these factors, but in the conditions of the present experiment and using hardened arachis oil as the form of fat, it was not appreciably operative. Burr and Burr used lard, and Evans and Burr lard, butter-fat, corn oil and coconut oil but no hardened fat, but Evans and Lepkovsky found that the sparing action of fat was also exercised by hardened cottonseed oil, so that it seems improbable that the virtue is lost in the process of hardening. The failure of fat to exercise its protective action against the development of scaly tail in the writers' experiments therefore remains unexplained, but it seems to exclude the hypothesis that scaly tail is the direct result of a pure fat deficiency.

SUMMARY.

1. A scaly tail condition, considered to be the same as that described by Burr and Burr and ascribed by them to lack of fat in the diet, was developed in the course of experiments in which the rats received diets with and without fat.

2. The condition was developed when the rats were kept on coarse wire grids, with highly purified caseinogen and usually when extracts of yeast, rather than whole yeast, were used as the source of B vitamins.

3. A comparison of the behaviour of rats housed on coarse and fine grids showed that the development of the condition was favoured by the use of the former.

4. The condition, when developed in a mild degree, could be cured by

the addition of whole dried yeast. Autoclaved yeast conferred no benefit, showing that the condition could not be due to an insufficiency of vitamin B₂.

5. The literature is discussed and it is suggested that the scaly tail condition may be correlated with some deficiency in the yeast vitamin complex and is influenced by the same factors as influence the action of the B vitamins, *i.e.* opportunities for coprophagy and the amount of fat in the diet.

The writers are indebted to the Medical Research Council for whole and half-time grants respectively and to Dr Harriette Chick for helpful criticism.

REFERENCES.

- Burr and Burr (1929). *J. Biol. Chem.* **82**, 345.
Drummond and Coward (1921). *Lancet*, ii, 698.
Evans and Burr (1928). *Proc. Soc. Exp. Biol. Med.* **25**, 390.
— and Lepkovsky (1929). *J. Biol. Chem.* **83**, 269.
Hindhede (1918). *Chem. Zentr.* **2**, 745.
Hume and Smith (1931). *Biochem. J.* **25**, 292.
McAmis, Anderson and Mendel (1929). *J. Biol. Chem.* **82**, 247.
Osborne and Mendel (1920–21). *J. Biol. Chem.* **45**, 145.

XXXVIII. THE MICRO-DETERMINATION OF BARIUM.

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(Received January 8th, 1931.)

IN the estimation of the barium present in barium hexosephosphates the ordinary macro-method on a small scale has been employed in this laboratory in the past. Sufficient of the salt to give about 20 mg. BaSO_4 was used, the barium precipitated in the boiling solution by the addition of very dilute sulphuric acid, and the barium sulphate filtered, ignited in the usual way and weighed on a micro-balance. The results obtained were often somewhat low and the agreement among duplicates was not always as good as was desirable. An investigation of the probable sources of error was made.

The error due to the use of coal gas for heating the barium sulphate.

It was found that heating the small platinum crucible to redness by means of a Bunsen burner to drive off the excess sulphuric acid after treatment of the incinerated residue with hydrochloric and sulphuric acids caused a considerable and variable loss in weight, due to reduction of some of the barium sulphate to barium sulphide in the presence of the hot, reducing gases from the burner. This loss can be eliminated by heating electrically at this stage, as shown by Table I.

Table I.

Weight of barium sulphate (mg.)	Manner of heating
17.200	Gas
21.300	Gentle gas heating, no blasting
20.205	Gas. " "
17.625	Blasting
16.850	"
18.275	"
19.395	Electrical
19.346	"
Another sample 18.654	Electrical
18.646	"
15.185	Gas
18.667	Electrical

Between each of the weighings recorded above the barium sulphate was treated with 4 drops of 2*N* HCl, followed by 4 drops of 2*N* H_2SO_4 and the moisture driven off by gently heating the crucible lid prior to heating, in the manner indicated in Table I, to drive off the sulphuric acid. In every case

after gas-heating there was a strong odour of H_2S on adding the hydrochloric acid, whereas after electrical heating there was no odour.

The error due to incomplete filtration.

It was found that loss of barium sulphate due to incomplete filtration could be eliminated by heating the precipitated barium sulphate and supernatant liquid for 6 to 8 hours at incipient boiling, in the usual manner, leaving at 0° overnight, filtering cold and re-filtering the filtrate through the same paper (Whatman 44). Previously, it had been found that on filtering hot and without re-filtration the loss from three barium determinations, each of which gave rise to about 18 mg. BaSO_4 , was 4.162 mg. This loss was determined by evaporating to dryness the combined filtrates from the three determinations and subtracting the weight due to solid impurities in the reagents employed. Barium chloride had been used as the source of barium so this was permissible.

In order to demonstrate the accuracy of the method the following experiment was performed. Approximately 10 mg. of recrystallised barium chloride were dissolved in 100 cc. of water. Six 2 cc. samples were taken with an Ostwald pipette. Three of these were treated according to Pregl's method for the micro-estimation of chlorine. The other three were treated according to the method to be described for the estimation of barium:

BaSO ₄ obtained, mg.	22.530	22.503	22.530
After re-treating with HCl and H ₂ SO ₄ and re-heating	22.535	22.515	22.530
Average	22.525 mg.		
AgCl obtained, mg.	27.720	27.760	27.715
Average	27.732		
Calculated as BaSO ₄	22.581 mg.		

The combined filtrates (ca. 100 cc.) from the three barium determinations were evaporated to dryness and the residue weighed. The same quantities of the same reagents used in the three barium determinations were evaporated to dryness and the residue weighed. Results:

Residue obtained from the three filtrates	1.268 mg.
Residue obtained from the reagents	1.054 mg.
BaSO ₄ in the combined filtrates	0.214 mg.
Loss of BaSO ₄ in each determination ($\frac{1}{3} \times 0.214$ mg.)	0.071 mg.

Thus, the weight of barium sulphate found agrees closely with the figure obtained by calculating the equivalent weight in barium sulphate of the silver chloride found, and the loss of barium sulphate is not more than 0.3 % of the total weight. This loss may be even less than that shown since any soluble impurity which may have been present in the recrystallised barium chloride could not be determined, and would, of course, be present in the residue obtained on evaporation of the combined filtrates. Also, agreement is found in the weights of barium sulphate before and after re-treatment with hydrochloric and sulphuric acids and re-heating. It may be noted that even when

evaporated to a small volume the combined filtrates from the three barium determinations showed no sign of precipitate even on standing for 24 hours.

Details of the method employed.

Place sufficient of the material to be analysed to give about 20 mg. BaSO_4 in a 150 cc. beaker, add water to 100 cc. and heat to incipient boiling. In a bulb of about 15 cc. capacity, which has been drawn down to a capillary at one end, place 10 cc. of water containing one drop of 2N H_2SO_4 and allow this solution to drip slowly into the solution in the beaker. Then add 1 cc. of 2N H_2SO_4 in 10 cc. of water in the same manner. Finally, add 1 cc. of 2N H_2SO_4 directly to the beaker. Cover the beaker with a clock-glass and maintain it at approximately 90° for 6 to 8 hours, thereby reducing the volume of the solution to about 20 cc. Set aside over night at 0° . Filter through a small filter-paper (4 to 5 cm.), stirring up the precipitate before each addition of liquid to the funnel. Never allow the surface of the liquid in the funnel to rise closer than 1 mm. to the top of the paper, thereby avoiding a ring of barium sulphate on the glass. When the liquid has all been filtered return the filtrate to the original beaker and repeat the filtration. In this way most of the precipitate is transferred to the paper. Transfer the remainder by using small portions of the filtrate. Finally, wash the beaker and precipitate with distilled water. Dry the filter-paper in the funnel in an oven at 100° and transfer it to a small, weighed platinum crucible. Char the paper slowly by heating the projecting lid only of the crucible with a small flame, never allowing the paper to burn. When charred, increase the heat gradually, finishing with strong heating to complete the incineration. Allow the crucible to cool. Add 2 drops of distilled water, making sure that the precipitate and filter ash are wet. (Otherwise, spattering will occur due to vigorous liberation of hydrogen sulphide on the addition of hydrochloric acid.) Add 1 drop of 2N HCl. Wait till the usual effervescence has moderated before adding 3 or 4 more drops. Add 4 or 5 drops of 2N H_2SO_4 . Drive off the moisture by heating the projecting lid only of the crucible with a very small flame, avoiding any approach to boiling. Finally, heat the crucible for 20 to 25 minutes on an electric heater to drive off the sulphuric acid. Placing the crucible as close as possible to a coil of red-hot resistance wire has been found to provide sufficient heat. It is advisable to remove the lid and heat it separately since, unless an electrically heated muffle is used, there is a tendency for the sulphuric acid to condense on the lid. Place the crucible on the copper block as used by Pregl. After 10 minutes transfer it to the micro-balance case and weigh it after a further 15 minutes. The empty crucible should, of course, have been weighed in the same manner. Repeat the operations from the point where the hydrochloric acid was added. The second weight should agree with the first within 0.03 mg.

XXXIX. THE METHYLATION OF HEXOSEMONOPHOSPHORIC ESTER.

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(Received January 5th, 1931.)

IN the original description of hexosemonophosphoric ester prepared by the action of yeast juice on fructose and glucose Robison [1922] expressed the opinion that the compound was probably a mixture of glucose- and fructose-monophosphoric esters. This conclusion was based on the properties of the dextrorotatory sugar obtained by acid hydrolysis of the ester and on the positive Selivanoff reaction for fructose given by all samples of the purified hexosemonophosphoric acid.

Meyerhof and Lohmann [1927] obtained further experimental evidence that the hexosemonophosphoric acid described by Robison was a mixture of an aldose- and a ketose-phosphoric ester. By estimating the reducing power according to Bertrand's method and comparing this figure with that obtained when using the Willstätter and Schudel [1918] hypiodite method they were able to calculate the percentage of aldosemonophosphoric ester present in the total hexosemonophosphoric acid. Lohmann [1928] attempted to separate these aldose and ketose constituents of hexosemonophosphoric ester by acid hydrolysis, since in the event of the ketosemonophosphoric acid present being identical with the fructosemonophosphoric acid prepared by Neuberg [1918] it should be more easily hydrolysed than the remaining aldosemonophosphoric acid. After partial hydrolysis Lohmann obtained an ester which showed an enhanced aldose content together with a specific rotation $[\alpha]_D^{18} + 35.7^\circ$ for the free acid. He did not succeed however in completely eliminating the ketose component from the aldosemonophosphate.

Robison and King [1929] by fractional crystallisation of the brucine salt of hexosemonophosphoric ester obtained a compound possessing a higher dextrorotation ($[\alpha]_{5461}^{20} + 21^\circ$ for the barium salt) than the original ester and an increased content of aldosemonophosphate (91 %) as measured by the iodimetric method modified according to Macleod and Robison [1929].

Towards the end of 1928 Robison and King had separated the aldosemonophosphate from yeast hexosemonophosphate and had obtained strong evidence that it was a monophosphoric ester of glucose. Their experiments yielded

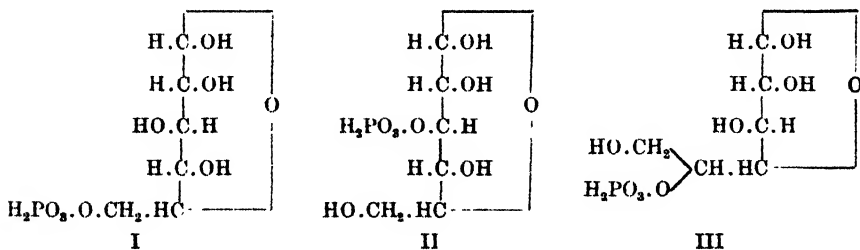
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sufficient of this purified aldosemonophosphate to enable work to be commenced on its methylation with a view to proving its structure.

The results of the methylation of this hexosemonophosphate were given in a short abstract by King and Morgan [1929, 1]. It was shown that if this hexosemonophosphate was methylated by Fischer's method, both at room temperature and at 60°, two distinct hexosides were formed. The hexoside produced at room temperature was rapidly hydrolysed by 0.1 *N* acid at 35°, the methyl-group being completely eliminated in about 24 hours. The hexoside produced by methylation at the higher temperature was not hydrolysed under these conditions. These two hexosides probably represent the reactive (furanose) and the stable (pyranose) forms whose formation could only take place if the hydroxyl-groups attached to the carbon atoms 4 and 5 are unsubstituted. It appeared therefore that the phosphate group was not in either of these positions. The earlier work of Robison [1922] had eliminated the possibility of the phosphoric acid group being attached in positions 1 and 2. There remained however positions 3 and 6. King and Morgan pointed out that while no definite conclusions could be reached as to the exact position of the phosphoric acid group, the rate of hydrolysis of this group pointed to position 6 as the most probable (I) but this inference appeared to be incompatible with the difference in the melting points of the osazones of hexosemonophosphoric acid and hexosediphosphoric acid which had been reported by Robison. It was therefore suggested [King and Morgan, 1929, 2] that the phosphoric acid group was in position 3 (II). Levene and Raymond [1929] having observed the formation of a reactive γ -glucoside when hexosemonophosphoric acid was methylated by Fischer's method at room temperature, concluded that this



phosphoric ester possessed an unstable furanose ring, the phosphoric acid group being attached to the fifth carbon atom (III). Methylation at these temperatures is, however, considered to favour the production of a γ -hexoside and, therefore, cannot be used as evidence that the phosphoric acid group is in the fifth position and has thus prevented the formation of the normal 1:5 ring.

Josephson and Proffe [1930] converted di-isopropylideneglucose by the action of phosphoryl chloride in pyridine solution at -18° to -20° into a di-isopropylideneglucose phosphate which on treatment with baryta and subsequent hydrolysis yielded a glucose-3-phosphate possessing a pyranose ring

structure. These authors claim that Robison's ester is not identical with their synthetic ester and, therefore, cannot be a glucose-3-phosphoric acid as suggested by King and Morgan.

The methylation experiments quoted in the original abstract of King and Morgan have been repeated using further preparations of purified aldosemonophosphate and the production of the two different types of hexosides has been confirmed. The evidence as to the nature of the oxide ring system in these two different types of hexosides has been further developed by comparing the rate of hydrolysis of the dephosphorylated methylhexosides with a methylhexoside of known structure. In the absence of any evidence which points to the migration of the phosphoric acid group during methylation it would appear highly probable that positions 4 and 5 in aldosemonophosphoric acid are not substituted by the phosphoric acid group. Meanwhile Robison and King [1929, 1931] isolated the pure aldosemonophosphoric acid component of hexosemonophosphoric acid and the determination of the melting point of its osazone showed this to be identical with that of the osazone of fructosemonophosphoric acid derived from fructosediphosphoric acid in which the phosphoric acid group is most probably in position 6 [Morgan and Robison, 1928; Morgan, 1929]. From this evidence the aldosemonophosphoric acid would appear to be an aldose-6-phosphoric acid and this conclusion is to some extent supported by the recent work of Levene and Raymond [1930]. These authors have also prepared the synthetic glucose-3-phosphoric acid and have shown that it differs markedly in properties from the Robison hexosemonophosphate. They have also prepared another synthetic ester which they consider to be glucose-6-phosphoric acid and which resembles the Robison ester in some though not in all respects [Robison and King, 1931].

EXPERIMENTAL.

The hexosemonophosphoric ester used in the methylation experiments was prepared by the fractional crystallisation from 20 % ethyl alcohol of the brucine salt of the crude ester isolated from the products of yeast juice fermentation, and a typical analysis of the purified barium hexosemonophosphate is given below.

Total phosphorus	7.6 %
Inorganic phosphate	Absent
Reducing power (H. and J.)	35.4 %
Iodine reduction (M. and R.)	39.3 % (\equiv 87 % aldose component)
$[\alpha]_{5461}^{18^\circ}$	+ 20.4°

METHYLATION OF THE HEXOSEMONOPHOSPHATE.

Methylation at 18°. Barium hexosemonophosphate (9.2 g.) was added to 200 cc. of anhydrous methyl alcohol containing hydrogen chloride in amount

calculated to give a final solution containing 0.5 % of free hydrogen chloride. To facilitate the rapid solution of the salt the mixture was shaken under conditions previously described in detail by Morgan [1927]. The barium salt dissolved rapidly to give a clear pale yellow solution and no separation of barium chloride occurred during the esterification. The progress of the esterification was followed by estimating the reducing power of the solution by the method of Hagedorn and Jensen and by frequent readings of the rotation in a 4 dm. polarimeter tube. During the first hour there was a sharp rise in the observed rotation followed by a sudden drop, the rotation continuing to decrease until esterification was complete. The course of this change is shown in Fig. 1, Curve A, while the analytical figures obtained during the esterifica-

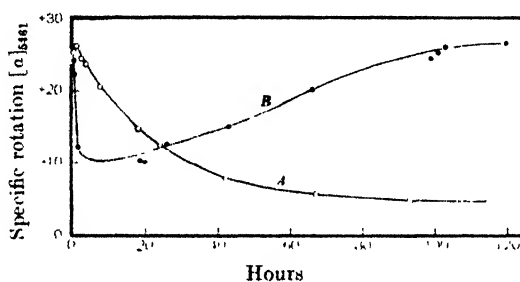


Fig. 1. Change in optical rotation during methylation of hexosemonophosphoric acid.
A. At 18°. B. At 60°.

Table I.

Time		Observed rotation $[\alpha]_{D441}$	Total phosphorus g.	Reducing power as glucose per 1 cc. of the solution mg.	Esterifi- cation %
Hrs.	Mins.				
	5	4.67°	0.692	14.8	0
	10	4.74	—	—	—
	25	4.77	—	—	—
	40	4.79	—	—	—
	55	4.81	—	—	—
1	25	4.67	—	—	—
2	10	4.49	—	—	—
3	30	4.36	—	—	—
7	25	3.79	0.691	11.70	21
18		2.72	0.694	9.40	36
24		2.72	—	—	—
42		1.43	0.685	6.45	56
67		1.03	0.682	5.35	64
93		0.88	0.687	3.95	73
115		0.82	0.689	3.05	79
160		0.79	0.690	2.70	85
211		0.63	—	1.32	91
*260		—	—	0.12	99

* After re-resolution in fresh methyl alcoholic hydrogen chloride.

tion are given in Table I. There was no formation of inorganic phosphate during the experiment. The reduction at the end of 8 days indicated that 90 % of the theoretical quantity of hexoside had been produced. The yield of methylhexosidemonophosphate was, however, considerably increased by

neutralising the 90 % equilibrium mixture, distilling off the methyl alcohol under reduced pressure at 35°–40°, and redissolving the hexose-hexoside mixture obtained in 200 cc. of dry methyl alcohol containing hydrogen chloride as described above. Under these conditions the solution became almost non-reducing (less than 1 %) and the methylhexosidemonophosphoric ester was obtained in an almost quantitative yield. The acid methyl alcohol solution of the hexoside was neutralised by the addition of silver carbonate, the precipitated silver chloride filtered off and the filtrate made just pink to phenolphthalein by the addition of methyl alcoholic solution of baryta. The precipitated barium methylhexosidemonophosphate was washed with anhydrous methyl alcohol and dried in a vacuum desiccator. The crude barium salt thus obtained was dissolved in 10 parts of water and fractionally precipitated with increasing concentrations of ethyl alcohol. The following fractions were collected:

Alcohol concentration	Weight of fraction
0–30 % (by volume)	3.3 g.
30–40 % "	2.1 g.
45–80 % "	4.5 g.

The optical rotation of these fractions showed no appreciable difference, thus indicating no separation into the α - and β -methylhexosidemonophosphates. That the α - and β -isomers were present seemed certain from the type of the methylation curve (Fig. 1, Curve A), the preliminary rise being due to the formation of a hexoside possessing greater dextrorotation than the original mixture of α - and β -hexosemonophosphoric acids. In the same way the subsequent fall in rotation presumably indicated the formation of the more laevorotatory isomer.

In another methylation under similar conditions 6.1 g. of barium hexosemonophosphate were dissolved in 300 cc. of anhydrous methyl alcohol containing hydrogen chloride. The reaction was stopped by the addition of excess barium carbonate when 93 % esterification had been reached. The solution was filtered and a hot saturated aqueous solution of baryta added to the filtrate until it was just pink to phenolphthalein. Two volumes of light petroleum and half a volume of ether were added, the resulting precipitate was collected by centrifuging and dried in a vacuum desiccator. The crude barium salt thus obtained, containing barium chloride as the principal impurity, was dissolved in 8 times its weight of water. The barium chloride was precipitated by the addition of a saturated aqueous solution of silver sulphate, the end-point being determined by centrifuging a small sample of the liquid and testing with silver sulphate solution. A slight trace of barium chloride amounting to, at most, 0.15 % of the total solid was left in the solution. The resulting precipitate was centrifuged off and washed with water. The large volume of centrifugate was reduced *in vacuo* over concentrated sulphuric acid to approximately the original volume, when a dark-coloured solution was obtained. To this solution slightly more than sufficient normal sulphuric acid

to give the free hexoside was added. The barium sulphate was quickly centrifuged off, the remaining trace of silver chloride being also removed. The clear colourless solution was then made pink to phenolphthalein by the addition of a cold saturated solution of baryta. The solution was then evaporated to the original volume and centrifuged to remove barium sulphate resulting from the slight excess of sulphuric acid. It was then treated with a mixture of alcohol, ether and light petroleum. The resulting precipitate, after drying over sulphuric acid *in vacuo*, gave the following analytical figures:

	% OCH ₃	% P	% Ba
Found	7.57	7.45	32.98
Calculated for C ₆ H ₁₀ O ₄ (OCH ₃)(PO ₄ Ba)	7.57	7.57	33.55

Methylation at 60°. Barium hexosemonophosphate (6.0 g.) was added to 100 cc. of anhydrous methyl alcohol containing hydrogen chloride. The general method and precautions observed were the same as those described above. A portion of the methyl alcoholic solution was at once transferred to a jacketed 4 dm. polarimeter tube which was kept, together with the remaining part of the solution, at 60° in an electric oven. At the end of the first hour barium chloride began to crystallise from the alcoholic solution, thus making it necessary to filter the solution before the polarimeter readings could be taken. The course of the methylation was followed and the resulting methylhexoside-monophosphate was isolated as described for the first methylation at 18°. The figures obtained are given in Table II. No inorganic phosphate was

Table II.

Time		Observed rotation [α] _{D441}	Total phosphorus g.	Reducing power as glucose per 1 cc. of the solution mg.	Esterification %
Hrs.	Mins.				
	12	5.22°	—	15.2	—
	40	4.79	0.430	10.8	29
1	20	2.62	—	—	—
18	30	2.20	—	5.0	67
20		2.15	—	—	—
26		2.71	—	—	—
41		3.25	0.410	2.3	85
66		4.35	—	1.1	93
99		5.25	—	—	—
101		5.45	—	—	—
103		5.62	0.408	0.6	96
120		5.75	0.410	—	—

liberated during the methylation. It will be seen that the curve showing change in rotation during methylation (Fig. 1, Curve B), is entirely different in form from the corresponding curve for the methylation at 18°. The curve shows a rapid fall during the first 20 hours, followed by a steady rise until the esterification is complete, the final value for the observed rotation being higher than the original value for the hexosemonophosphoric acid. In a second methylation at 60° 6.1 g. of barium hexosemonophosphate were dissolved in 300 cc. of anhydrous methyl alcohol containing hydrogen chloride. The separation of the hexoside and its purification by means of silver sulphate were carried out

in the manner already described under the methylation at 18°. On analysis the methoxyl values were found to be higher than that calculated for barium methylhexosidemonophosphate. It was thought that this might possibly be due to partial methylation of the phosphate group, although potassium dihydrogen phosphate under the same conditions showed no evidence of esterification, a result in agreement with that obtained by Morgan [1927]. The brucine salt was prepared and was repeatedly recrystallised from aqueous alcohol and thereafter reconverted into the barium salt. The methoxyl content of the latter, though somewhat lower than before, was still higher than the calculated figure. Under the conditions employed it is possible that the dimethylacetal derivative would also be formed and might account for the high methoxyl value obtained. The regenerated barium salt gave the following analytical results:

	% OCH ₃	% P	% Ba
Found	8.23	7.49	32.76
Calculated for C ₆ H ₁₀ O ₄ (OCH ₃)(PO ₄ Ba)	7.57	7.57	33.55

ACID HYDROLYSIS OF THE METHYLHEXOSIDEMONOPHOSPHATES.

Hexosidemonophosphate formed at 18°. The barium methylhexosidemonophosphate (47.4 mg.) was dissolved in 10 cc. of 0.1 N HCl for the determination of the rate of elimination of the methyl group at 37°. The extent of hydrolysis was measured from time to time by estimating the reducing power of the solution by the Hagedorn and Jensen method. The results shown in Fig. 2,

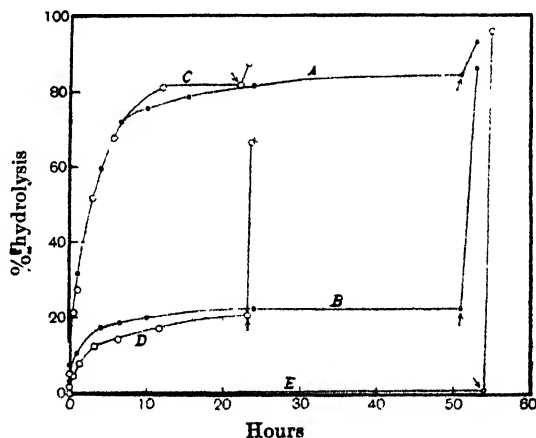


Fig. 2. Hydrolysis in 0.1 N HCl at 37°.

- A. Methylhexosidemonophosphate from methylation at 18°.
- B. Methylhexosidemonophosphate from methylation at 60°.
- C. Dephosphorylated methylhexosidemonophosphate from methylation at 18°.
- D. Dephosphorylated methylhexosidemonophosphate from methylation at 60°.
- E. α -Methylglucoside.

→ At these points the solutions were made N with HCl and heated at 100° for the times indicated.

Curve A, indicate that hydrolysis was almost complete at the end of 24 hours. This was confirmed by raising the concentration of HCl to N and heating at

100°, when the reducing power, expressed as % of the barium methylhexoside originally present, was increased only from 37.2 to 40.9.

Hexosidemonophosphate formed at 60°. The barium methylhexosidemonophosphate (51.1 mg.) was dissolved in 10 cc. of 0.1 *N* HCl and kept at 37°, the rate of removal of the methyl group being measured as in the previous acid hydrolysis. Curve B, Fig. 2 shows that a small but rapid rise in the reducing power occurred during the first few hours; this was possibly due to the presence of the dimethylacetal derivative mentioned above together with any of the more reactive hexosides which may have been produced during the methylation. No further increase was observed after 24 hours and the reducing power at this point indicated that only 22 % of the total hexoside had been hydrolysed. This amount presumably includes the dimethylacetal derivative, which by calculation from the methoxyl content may have been present to the extent of 20 %. At the end of 50 hours the methylhexoside solution was made *N* with hydrochloric acid and heated at 100°, and after this treatment the reducing power was found to be very nearly equal to that calculated assuming complete hydrolysis of the hexoside.

These experiments indicate that the hexoside produced by methylation at 60° is not hydrolysed to any appreciable extent under conditions which bring about almost complete hydrolysis of the methylhexosidemonophosphate produced at 18°.

THE ENZYMIC DEPHOSPHORYLATION OF THE METHYLHEXOSIDEMONOPHOSPHATES.

Methylhexosidemonophosphate formed at 18°. A sample of the purified barium methylhexosidemonophosphate (0.503 g.) was dissolved in water and the equivalent amount of potassium sulphate added. The barium sulphate was removed by centrifuging and the solution of the potassium salt adjusted to p_H 8.4 with a dilute solution of potassium hydroxide. The bone phosphatase preparation, 100 mg. [Martland and Robison, 1929], was then added together with a few drops of chloroform, the p_H again adjusted to 8.4 and the enzymic hydrolysis allowed to proceed at room temperature. Estimations of inorganic phosphate showed that 91 % of the phosphorus was eliminated at the end of 72 hours and that no further hydrolysis occurred in a further 24 hours. At this stage the solution was treated with four times its volume of alcohol, and after filtration the alcoholic solution was evaporated to dryness. The residue was extracted with boiling ethyl alcohol and the alcoholic extract filtered and evaporated. The resulting syrup was then extracted with a small quantity of water, filtered and again evaporated to dryness, when it yielded 210 mg. of a syrup which was free from phosphorus and possessed a reducing power of only 4.5 %. After drying over phosphorus pentoxide *in vacuo* the methoxyl was determined:

Found	14.67 %
Calculated for $C_6H_{11}O_8(OCH_3)$	15.98 %

Methylhexosidemonophosphate formed at 60°. The purified barium methylhexosidemonophosphate (0.638 g.) was converted to the potassium salt and treated with 500 mg.¹ of the bone phosphatase preparation under conditions similar to those described above. At the end of 24 hours 93.5 % of the phosphorus was eliminated and no further hydrolysis took place during the next 24 hours. The dephosphorylated hexoside was then isolated and purified as described above. Analysis for methoxyl gave the following results:

Found	12.73 %
Calculated for $C_6H_{11}O_5(OCH_3)$	15.98 %

The methoxyl value indicates that the product contained a considerable proportion of contaminating material which possibly may have been derived from the phosphatase preparation of which an unnecessarily large amount was used for the hydrolysis.

THE ACID HYDROLYSIS OF THE DEPHOSPHORYLATED METHYLHEXOSIDEMONOPHOSPHATES.

A carefully dried sample of the dephosphorylated methylhexosidemonophosphate (0.114 g.) produced by methylation at 18° was dissolved in 40 cc. of 0.1*N* HCl and kept at 37°. The extent of the removal of the methyl group was measured by estimating the reducing power of the solution by Hagedorn and Jensen's method as in the acid hydrolysis of the methylhexosidemonophosphate. The optical rotation of the solution in a 4 dm. tube was observed during the hydrolysis. The results are shown in Fig. 2, Curve C; Fig. 3, Curve A;

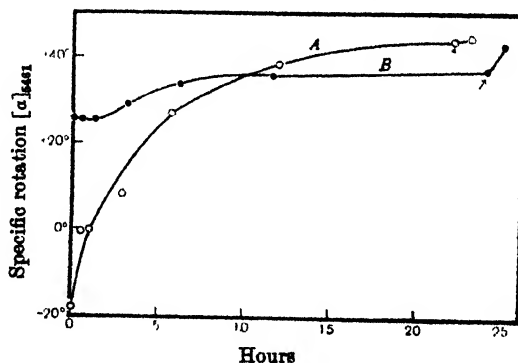


Fig. 3. Change in optical rotation during hydrolysis in 0.1*N* at 37°.

A. Dephosphorylated methylhexosidemonophosphate from methylation at 18°.

B. Dephosphorylated methylhexosidemonophosphate from methylation at 60°.

→ At these points the solutions were made *N* with HCl and heated at 100° for 1 hour.

and in Table III, and indicate the extreme ease with which the methyl group is eliminated under these conditions. About 80 % hydrolysis was effected in 12 hours and this figure was not increased in the following 12 hours. Heating at 100° for 1 hour in *N* HCl increased the hydrolysis to 87 %. At the end

¹ This amount was used in error, in place of 100 mg.

Table III.

Time		% hydrolysis (H. and J.)	Observed rotation $[\alpha]_{5461}$
Hrs.	Mins.		
0	0	4.87	-0.21°
*0	30	21.29	-0.06
1		27.30	-0.04
2	52	51.70	+0.09
5	44	67.77	+0.30
12		81.32	+0.43
22	15	81.81	+0.47
†23	15	87.72	+0.36

* 39 cc. of the original solution + 0.4 cc. 10 *N* HCl + 0.6 cc. H₂O.

† After heating 30 cc. of the above solution + 8 cc. 10 *N* HCl + 2 cc. H₂O at 100° for 1 hour.

of the hydrolysis at 37° the specific rotation, based on the weight of material taken was $[\alpha]_{5461} + 44.0^\circ$, and on the glucose present as indicated by Hagedorn and Jensen's method was $[\alpha]_{5461} + 55.6^\circ$. After hydrolysis at 37° the reduction given by the iodimetric method of Macleod and Robison was 77.7 %.

Table IV.

Time		% hydrolysis (H. and J.)	Observed rotation $[\alpha]_{5461}$
Hrs.	Mins.		
0	0	1.8	+0.30°
*0	30	4.5	+0.27
1	15	7.7	+0.27
3	10	12.4	+0.32
6	15	14.2	+0.36
11	40	17.1	+0.38
23		20.5	+0.395
†23	30	66.5	+0.40
‡24		67.6	+0.36

* 36.5 cc. of the original solution + 0.4 cc. 10 *N* HCl + 3.1 cc. H₂O.

† After heating 35.2 cc. of the above solution + 4 cc. 10 *N* HCl + 0.8 cc. H₂O at 100° for 30 minutes.

‡ 30 minutes' further heating at 100°.

A sample of the dephosphorylated methylhexoside (0.116 g.) obtained from the product of methylation at 60° was dissolved in water, made up to 40 cc. and the reducing power and rotation of the solution were determined. The course of the hydrolysis was followed by frequent estimations of the reducing power (Hagedorn and Jensen) of the solution and by the change in optical rotation as observed in a 4 dm. tube. Fig. 2, Curve D shows that at the commencement of the hydrolysis there was a rapid but limited rise in the reducing power of the solution indicating the presence of about 20 % of a substance readily hydrolysed by 0.1 *N* HCl at 37°. After 20 hours, as no further hydrolysis appeared to take place, the solution was made *N* with hydrochloric acid and heated at 100° for half an hour. This procedure raised the percentage hydrolysis from 20.5 to 66.5, while after a further 30 minutes at 100° this figure was only increased to 67.6. The iodine reduction value determined at this stage corresponded with 74.9 % hydrolysis. The specific rotation after 30 minutes' heating at 100° calculated on the basis of the glucose present (Hagedorn and Jensen) was $[\alpha]_{5461}^{30^\circ} + 69.6^\circ$ and after a further period of

30 minutes at 100° , $+61.5^{\circ}$. The change in optical rotation of the solution during hydrolysis is shown in Fig. 3, Curve B.

The dephosphorylated methylhexosides derived by enzymic hydrolysis from the products of methylation of aldosemonophosphate at 18° and 60° respectively exhibit the same pronounced difference in their rates of hydrolysis by $0.1N$ HCl at 37° as do the methylhexosidemonophosphates themselves. It is clear from the curves that the presence of the phosphate group does not materially affect the rate of hydrolysis of the methoxyl group.

In order to compare the rates of hydrolysis of these hexosides with that of a compound of known constitution a sample of recrystallised α -methylglucoside was hydrolysed by $0.1N$ HCl at 37° followed by N HCl at 100° . The results are shown in Fig. 2, Curve E. The reducing power and the specific rotation of the hydrolysis product were determined in a separate experiment in which 0.1327 g. of α -methylglucoside in 50 cc. of $2N$ HCl was heated at 100° for 1 hour. Calculated from the reducing power determined by the Hagedorn and Jensen method, the hydrolysis amounted to 96.5% , and the specific rotation $[\alpha]_{5461}^{20^{\circ}}$ of the product was $+65.9^{\circ}$. The reducing power by the iodimetric method, however, indicated complete hydrolysis and on this basis the specific rotation was $+63.4^{\circ}$. These values for the specific rotation are somewhat higher than that of glucose in aqueous solution ($[\alpha]_{5461}^{20^{\circ}} + 62.5^{\circ}$), but a determination of the specific rotation of glucose in similar low concentration in $2N$ HCl gave the value $+63.6^{\circ}$, while after heating for half an hour at 100° the reducing power by the Hagedorn and Jensen method showed that some destruction of glucose had taken place (3%) and the specific rotation, calculated from this reducing power, was $+66.3^{\circ}$.

SEPARATION OF THE α - AND β -METHYLHEXOSIDEMONOPHOSPHATES.

Small amounts of the barium methylhexosidemonophosphates produced by methylation at 18° and 60° respectively were converted into the brucine salts. The barium was removed by shaking an aqueous solution of these salts with a slight excess of brucine sulphate until the reaction was complete. By allowing the alcoholic solutions of the brucine salts to evaporate slowly several crops of crystals were removed. From the brucine salt of the methylhexosidemonophosphoric ester methylated at 18° crops of crystals yielded, on converting to the barium salt, non-reducing compounds having extreme specific rotations of $[\alpha]_{5461}^{20^{\circ}} + 13^{\circ}$ and $+49^{\circ}$, while the brucine salts of the more stable hexoside yielded non-reducing barium salts possessing specific rotations of $[\alpha]_{5461}^{20^{\circ}} + 3.5^{\circ}$ and $+39^{\circ}$. These figures indicate a separation into the α - and β -isomers of the corresponding reactive and stable hexosides, but since the brucine salts were not exhaustively fractionated using different solvents it is not possible to claim that the specific rotations are those of the pure isomers.

DISCUSSION.

The simplest and most probable explanation of the experimental results described in this paper is that during the methylation of purified aldosemonophosphate two types of aldoses are formed presumably containing a reactive (furanose) or a stable (pyranose) ring structure according to the temperature of methylation. This explanation assumes that only furanose and pyranose ring structures are formed; this assumption is, however, strongly supported by a large mass of experimental work in the sugar group. At present there is no authentic case of a propylene oxide (1:3) sugar known. The structure of di-*isopropylidene*xyllose has been investigated by Haworth and Porter [1928] who originally considered that this compound presented a unique opportunity to investigate the possible existence of a propylene oxide (1:3) ring system. They concluded, however, after careful examination, that this compound possesses a furanose (1:4) ring and thus falls into line with one of the two known oxide ring systems. It would appear, therefore, that in the absence of any evidence indicating that the two hexosides produced from the purified aldosemonophosphate by simple methylation at 18° and 60° are different from the known furanose and pyranose types, we may conclude that the phosphoric acid group is not attached at the 4th or 5th position in the aldosemonophosphate.

It may be pointed out that although the ester used in these experiments contained only 87 % of the pure aldosemonophosphate, since isolated by Robison and King [1931], the presence of the second component could not possibly account for the formation of the two methylhexosides in the yields obtained.

The recent work of Robison and King on the melting point of the osazone of the purified aldosemonophosphate and on the oxidation of this ester points to the phosphoric acid group being attached in position 6, while from the synthetic side the evidence of Josephson and Proffe seems to eliminate the possibility of the phosphoric acid being in position 3, a conclusion which is also supported by Levene and Raymond. On the evidence now available it would therefore appear probable that the aldosemonophosphate component of Robison's original hexosemonophosphate is glucose-6-phosphoric acid.

SUMMARY.

1. The methylation of the aldosemonophosphate component of Robison's fermentation hexosemonophosphate by Fischer's method at 18° and at 60° has yielded two distinct forms of methylhexosidemonophosphate.
2. The methylhexosidemonophosphate produced by methylation at 18° is extremely sensitive to acids. With 0.1 *N* acid at 36° it is rapidly hydrolysed yielding a reducing hexosemonophosphoric acid.
3. The corresponding hexoside produced by methylation at 60° is stable in the presence of 0.1 *N* acid at 37°. Its rate of hydrolysis closely resembles that of α -methylglucoside under the conditions described.

4. The bearing of these experimental results on the constitution of the aldosemonophosphate component of Robison's fermentation hexosemonophosphoric ester is discussed.

In conclusion we wish to express our gratitude to Dr R. Robison for his very helpful criticism and advice.

REFERENCES.

- Haworth and Porter (1928). *J. Chem. Soc.* 611.
 Josephson and Proffe (1930). *Liebig's Ann.* **481**, 91.
 King and Morgan (1929, 1). *Chem. Ind.* **48**, 143.
 ——— (1929, 2). *Chem. Ind.* **48**, 296.
 Levone and Raymond (1929). *J. Biol. Chem.* **81**, 279.
 ——— (1930). *J. Biol. Chem.* **89**, 479.
 Lohmann (1928). *Biochem. Z.* **194**, 306.
 Macleod and Robison (1929). *Biochem. J.* **23**, 517.
 Martland and Robison (1929). *Biochem. J.* **23**, 237.
 Meyerhof and Lohmann (1927). *Biochem. Z.* **185**, 113.
 Morgan (1927). *Biochem. J.* **21**, 675.
 ——— (1929). *Chem. Ind.* **48**, 143.
 ——— and Robison (1928). *Biochem. J.* **22**, 1270.
 Neuberg (1918). *Biochem. Z.* **88**, 432.
 Robison (1922). *Biochem. J.* **16**, 809.
 ——— and King (1929). *Chem. Ind.* **48**, 143.
 ——— (1931). *Biochem. J.* **25**, 323.
 Willstätter and Schudel (1918). *Ber. deutsch. chem. Ges.* **51**, 780.

XL. HEXOSEMONOPHOSPHORIC ESTERS.

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THE hexosemonophosphoric ester isolated from the products of the fermentation of glucose and fructose by yeast juice [Harden and Robison, 1914; Robison, 1922] was considered, from its properties and behaviour on hydrolysis, to be a mixture of isomers, probably the monophosphoric esters of glucose and fructose. The differences observed in the specific rotations of specimens prepared from different fermentation experiments suggested that these isomers occurred in varying proportions.

The presence of aldose and ketose derivatives in this ester was confirmed by Meyerhof and Lohmann [1927], who compared its reducing power as estimated by Bertrand's method with the value found by the iodimetric method of Willstätter and Schudel [1918] and, from the ratio of these values, calculated that a sample of the ester contained 17 % of the fructose derivative. A higher percentage of the ketose component can, however, be deduced from their data if the calculation is based on the Willstätter number, *i.e.* the ratio of total hexose to the iodimetric value. While the reducing power of these esters towards Bertrand's copper solution is always lower than that of the unsubstituted hexose, the reducing power of an aldosephosphate towards hypiodite should be the same as that of the free aldose, a conclusion supported by Sobotka's [1926] experiments with methylated glucoses and by our present results. On this basis Meyerhof and Lohmann's figures indicate the presence of only 66 % and 68 % aldosemonophosphate in two of their preparations and 57 % in a preparation obtained from this laboratory. The last figure is in agreement with our own value (55 %) for the same preparation (see Table I, No. 1). Lohmann [1928] provided further evidence for the dual nature of the ester by investigating the kinetics of its hydrolysis in *N* HCl. Having shown that the rate of hydrolysis falls considerably after a certain period he made use of this method to remove as much as possible of the more easily hydrolysable ketose ester, thereby raising the proportion of aldosemonophosphate from 68 % to 79 % (based on the Willstätter number) and the $[\alpha]_D$ of the free ester from + 28.5° to + 35.7°. Lohmann concluded that either his residual ester must still contain non-aldose components or the aldehyde oxygen must be linked in two different rings, one of which is not split by hypiodite.

Similar difficulties have been encountered in our own attempts, continued over several years, to separate the components of hexosemonophosphoric

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ester. A knowledge of the constitution of these components was much to be desired but it was first of all essential to isolate them in reasonably pure condition. The methods employed for this purpose have included the fractional crystallisation of the brucine salt, fractional precipitation of normal and basic barium and lead salts and fractional hydrolysis. Two years ago we had obtained a crystalline brucine salt, which, after many recrystallisations, had the appearance of homogeneity. The corresponding barium salt had $[\alpha]_{5461} + 21^\circ$, but the iodimetric reducing power was still 9 % below that required for a pure aldosemonophosphate. Evidence was obtained that this ester was a derivative of *d*-glucose [Robison and King, 1929] and its methylation was commenced in order to determine the position of the phosphoric acid group [King, McLaughlin and Morgan, 1931]. Work on the further purification of the ester was, however, continued and at length yielded a compound whose iodimetric value corresponded exactly with that required for a pure aldosemonophosphate. The preparation and investigation of this ester and of the other components of hexosemonophosphate are here described.

Preparation of the hexosemonophosphoric esters.

The methods now employed for the preparation of the phosphoric esters and for their preliminary separation according to the solubility of their barium salts have been described in another communication [Robison and Morgan, 1930] in which a general account was given of the quantitative examination of the separate fractions. The material for the present investigation of the monophosphoric esters was provided by the soluble-B fractions from the fermentation of glucose or fructose with yeast juice. These fractions were obtained by addition of basic lead acetate to the 10 % alcohol filtrate after removal of the sparingly soluble barium salts, reconversion of the basic lead salt into the barium salt and extraction of the latter with 10 % alcohol. The soluble-B fractions were in some cases converted directly into the brucine salt, but usually a further purification was first carried out by dissolving them in 10 % alcohol and treating the solution with mercuric acetate as previously described [Robison, 1922]. The monophosphate was reprecipitated as the basic lead salt, which was converted back into the barium salt and purified by solution in 10 % alcohol and reprecipitation with alcohol.

Isolation of the aldosemonophosphoric ester.

Crystallisation of the brucine salt. The barium salt was dissolved in 5 times its weight of water and treated with the amount of 5*N* H₂SO₄ required to precipitate the whole of the barium. The barium sulphate was removed by centrifuging and washed once with water and 3 times with 96 % alcohol. The acid solution was treated with a slight excess of brucine (2.2 g. for each cc. 5*N* H₂SO₄), rather less than half of this amount being added in solid form before centrifuging, the remainder, dissolved in hot 50 % alcohol, after the

final washing was completed. Crystallisation of the brucine salt usually commenced immediately, but the solution was allowed to remain at 0° for at least a week before filtration. The salt was recrystallised by dissolving it in 7 or 8 times its weight of 20 % ethyl alcohol at 40° and allowing the filtered solution to remain at 0°. Two types of crystals were obtained, the first fractions consisting of hard, translucent needles, while later fractions, which separated on allowing the mother-liquors to evaporate spontaneously, consisted partly of needles and partly of hexagonal plates. The separate fractions were repeatedly recrystallised from 20 % alcohol and portions of each were then converted into barium salts by dissolving in 20 % alcohol, adding a solution of barium acetate in slight excess and precipitating the barium hexosemonophosphate with absolute alcohol. The salts were purified several times by solution in water and reprecipitation with absolute alcohol. Analyses showed that the barium salts obtained from the needle fractions contained an increased proportion of aldosemonophosphate while those obtained from the hexagonal plates contained less than 50 % of the aldose component. It was noticed that on recrystallisation of the platelets some clusters of needles were usually obtained, and it was concluded that these two types of crystals represented mixed brucine salts of the aldose- and ketose-monophosphates in different proportions. One of the needle fractions obtained after exhaustive fractionation gave a barium salt, which, by iodimetric estimation, contained 91 % of aldose ester, but, in general, recrystallisation from 20 % ethyl alcohol failed to yield so pure a product. A brucine salt recrystallised 4 times from this solvent, the solution being left at 0° for at least a week at each recrystallisation in order to obtain the maximum crop of crystals, contained only 79 % of the aldose component. The next stage in the purification was effected by recrystallisation of this brucine salt from boiling 90 % or 95 % methyl alcohol, from which it slowly separated, at room temperature or at 0°, in large clumps of soft, opaque, silky needles. After two further recrystallisations a portion of the salt was converted to barium salt, the analysis of which showed that the proportion of aldose ester had increased from 79 % to 90 %. The specific rotation was, however, somewhat lower than that of the 91 % salt mentioned above. The final stage in the purification of the aldosemonophosphate was achieved by recrystallisation from boiling absolute methyl alcohol in which the salt is only sparingly soluble. A small amount of sparingly soluble residue was filtered off and a small fraction of hard, minute crystals which separated rapidly on cooling to room temperature was also removed. The clear solution was then left in a closed vessel at 0° for some days, during which almost the whole of the remaining brucine salt crystallised out. These operations were repeated twice but in the last recrystallisation the crystals were removed by filtration after 24 hours. This fraction and the salt remaining in the mother-liquors were converted into the barium salts which were purified as already described and analysed. The iodimetric value of the salt prepared from the crystals was found to be equal to that required for 100 % aldosemonophos-

phate, while the barium salt prepared from the mother-liquors was of almost equal purity.

Fractional hydrolysis. Lohmann's method of separation of the esters by fractional hydrolysis was also tried but, as in his experiments, with only partial success. For this attempt we used a sample of hexosemonophosphate that had already been purified by recrystallisation of the brucine salt from 20 % ethyl alcohol and contained 80 % of the aldose derivative. The barium salt was hydrolysed with N H_2SO_4 for 6 hours at 100° , 14 % of the phosphate being set free. The residual monophosphate now contained 84 % of the aldose ester. After a further 6 hours in N H_2SO_4 at 100° the proportion of aldose ester had increased to 88 % and rose to 91 % after a third hydrolysis of similar duration. During the 18 hours 35 % of the original ester had been hydrolysed.

Other components of hexosemonophosphoric ester.

The dual nature of the platelet fractions obtained during the crystallisation of the brucine salt from 20 % ethyl alcohol was confirmed by submitting the barium salts derived from these fractions to bromine oxidation. The salts, dissolved in water, were treated with bromine and barium carbonate at room temperature. The flask was shaken at intervals during 24 hours and the excess of bromine then removed by aeration. The filtered solution was made acid to methyl orange and treated with 4 times its volume of alcohol by which the acid salt of the oxidation product (phosphohexonic acid) was precipitated. The filtrate was neutralised to phenolphthalein with baryta and the precipitated barium hexosemonophosphate filtered off, purified by solution in 10 % alcohol and reprecipitation, and analysed. After a second treatment with bromine the purified barium salt had a very low iodimetric value and specific rotation, and was very similar in most respects to Neuberg's fructosemonophosphate. For the purposes of comparison a specimen of the latter ester was prepared by partial hydrolysis of hexosediphosphate which had been very carefully purified by precipitation of the barium salt from its aqueous solution by heating the latter to 75° . This purification was very essential since the diphosphate is likely to be contaminated with small amounts of aldosemonophosphate carried down as the double barium salt [v. Robison and Morgan, 1930]. Such aldosemonophosphate is not appreciably hydrolysed during the short treatment with acid and therefore becomes concentrated in the fructosemonophosphate produced. The chief differences between the ester derived from the platelet fraction and the Neuberg ester lay in the lower Hagedorn and Jensen reducing power of the former, which may indicate that it was contaminated with the unknown ester referred to below.

The final mother-liquors from the crystallisation of the brucine salts in 20 % alcohol still contained a considerable amount of phosphoric ester, very soluble in this solvent and even in a much higher concentration of alcohol. The analyses of the barium salts prepared from these mother-liquors indicated the presence of some unknown ester since the analytical data could not be

reconciled with any possible mixture of the four known esters. The specific rotations of these salts were higher than that of the aldosemonophosphate, but this might be due to the presence of a very small proportion of trehalosemonophosphate. Whether these salts contain the new ester in small or large proportion cannot be decided until it has been isolated and its properties investigated. In spite of much work this has not yet been achieved. A comparison of the Hagedorn and Jensen values found for the original hexosemonophosphate (*cf.* Table I, salts 1–4) with those of the pure aldose and ketose esters suggests, however, that the unknown ester is present in significant amounts.

Analyses of the various barium salts obtained in these operations and referred to in the above paragraphs are shown in Table I. One of the original specimens of hexosemonophosphate prepared 10 years ago from glucose [Robison, 1922, Table II, No. 7], has also been analysed by the micro-methods now employed and the results are included in Table I. Details of these methods have already been given, but some notes may be added here. The estimations were carried out on the substance dried over sulphuric acid, the moisture being separately estimated in Pregl's micro-drying apparatus. The results are calculated for the anhydrous salt. The values quoted for "fructose" were found by estimating the colour developed with the Selivanoff reagent and do not represent the actual percentage of this hexose in the sample. Thus, the value for barium fructosemonophosphate is only half the percentage of hexose in the salt.

The estimation of reducing power by the Hagedorn and Jensen method was carried out with addition of 0.5 cc. $N/2$ sodium hydroxide to each tube. Without this addition the curve of reducing power for these esters deviates so widely from the curve determined for glucose that very inconsistent values are obtained by reference to the glucose table. Even with this extra alkali the percentage reducing power is found to vary a little according to the amount of ester taken. For both glucose and fructosemonophosphates the values lie between 35 % and 37 %, that is, the reducing power is about 80 % of that of the unsubstituted hexose.

In the iodimetric estimations, carried out by the method of Macleod and Robison [1929], it was found that the aldosemonophosphate was even more sensitive than glucose to various conditions, particularly to the concentration of alkali. Low results were obtained unless the iodine was in very large excess or if the amount of 5 % sodium carbonate solution was increased from 0.2 cc. to 0.4 cc., an amount shown to be permissible in the estimation of glucose.

The specific rotation of barium aldosemonophosphate varies slightly with the concentration of the salt and still more with changes in p_H of the solution. Thus, the value of $[\alpha]_{5461}$ for salt No. 11 in 0.5 % solution (p_H *circ.* 8.3) was +19.6°, at p_H 9.8 it was +18.0°, while at p_H 5.8 it was +23.3°. The free acid corresponding to this salt had $[\alpha]_{5461}$ +40.0°, but in 0.07 N H_2SO_4 this

became + 43.6°. These differences are possibly due to changes in the equilibrium between the un-ionised ester and its ions.

Table I. *Analyses of barium salts of hexosemonophosphoric acids obtained from the products of fermentation by yeast juice.*

No. of salt	Source of the barium salt	P %	Fructose (Seli-vanoff) %	Reducing power as glucose (%)		Aldose-mono-phosphate %	[α] ₅₄₆₁ ^{20°}	[α] ₅₄₆₁ ^{20°} of free acid
				H. and J.	Iodine			
1	"Hexosemonophosphate" [Robison, 1922]	7.85	6	30.0	25.2	55	+ 14.4°	+ 29.5°
2	Soluble-B fraction	7.37	9	27.4	21.1	16	+ 11.3°	—
3	"	7.45	—	30.8*	27.7	61	+ 15.0°	—
4	Soluble-B after further purification by mercuric acetate and basic lead precipitation	7.90	5	31.9	23.6	52	+ 16.3°	—
5	Brucine salt after repeated fractional crystallisation from 20 % ethyl alcohol. Hard, translucent needles	7.80	—	31.8*	41.4	91	+ 21.0°	—
6	Brucine salt recrystallised 4 times from 20 % ethyl alcohol. Hard needles	7.84	0.5	31.9	36.1	79	+ 17.9°	—
7	Brucine salt recrystallised 4 times from 20 % ethyl alcohol, 3 times from 90 % methyl alcohol. Opaque, silky needles	7.86	0.5	36.1	41.0	90	+ 19.2°	—
8	Brucine salt recrystallised twice from 20 % ethyl alcohol, 4 times from 95 % methyl alcohol, 3 times from absolute methyl alcohol. Opaque, silky needles	7.88	0.5	35.5	45.7	100	+ 20.6° ($n_D^{20} = 1.461$) + 21.2° ($n_D^{20} = 1.461$)	+ 41.4° ($n_D^{20} = 1.461$)
9	Mother-liquors in final recrystallisation of above brucine salt	7.87	0.5	35.3	44.9	99	+ 20.1° ($n_D^{20} = 1.461$)	—
10	Brucine salt recrystallised from 20 % ethyl alcohol	7.90	2.4	35.1	36.6	80	+ 16.2°	—
11	Residual salt after 18 hrs. hydrolysis of No. 10, with $N H_2SO_4$ at 100°	7.90	9.0	34.8	41.4	91	+ 19.0°	+ 40.0°
12	Brucine salt fractionally crystallised from 20 % ethyl alcohol. Hexagonal plates	7.76	—	25.4	18.9	41	+ 8.2°	—
13	Residual salt after oxidation of No. 12 with bromine and removal of phosphohexonate	7.75	22	27.6	2.6	6	+ 2.1°	—
14	Residual salt after oxidation of another similar fraction	7.60	20	29.0	1.3	3	+ 3.3°	—
15	Fructosemonophosphate (Neuberg) prepared from highly purified hexosediphosphate	7.80	22	36.2	3.0	7	+ 0.7°	—
16	Final mother-liquors in the recrystallisation of brucine salts	7.05 6.90	6.8 5.7	25.3 25.0	16.7 21.1	37 46	+ 21.4° + 22.2°	—

$C_6H_{11}O_6PO_4Ba$ requires P = 7.85 %; $C_6H_{12}O_6$ = 45.6 %.

* Determined without addition of NaOH.

Osazones of hexosemonophosphoric esters.

The osazone of hexosemonophosphoric ester was originally described [Robison, 1922] as melting at 139°, and it was concluded that this osazone was not identical with that prepared by Young [1911] from Harden and Young's fructosediphosphoric ester. Should the aldose ester prove to be a derivative of glucose, or of mannose, the non-identity of the osazones would show that the phosphoric acid group is not in the same position in the two esters. The demonstration of the composite nature of the original hexosemonophosphate had, however, thrown doubt on the purity of the osazone

prepared from it and a re-examination of this point was, therefore, necessary. Osazones were prepared from the pure aldosemonophosphate (No. 8, Table I) and from the Neuberg fructosemonophosphate (No. 15) prepared from hexosediphosphate. Solutions of the free acids, prepared from the barium salts, were treated with as much phenylhydrazine as they would dissolve before adding the calculated amount of the base in acetic acid. The solutions were heated in a boiling water-bath for short periods ($\frac{1}{2}$ hour), cooled to 0° , filtered and again heated. The osazones were recrystallised by dissolving in boiling alcohol and adding chloroform to the filtered solution. For the determination of the melting points a short thermometer, with the mercury thread completely immersed in the bath, was used and the melting points are, on this account, higher than that given by Young ($151\text{--}152^\circ$). The tube containing the osazone was introduced when the temperature of the bath was 4° below the melting point and was rising at the rate of $6\text{--}8^\circ$ per minute. The osazones from the aldosemonophosphate and from fructosemonophosphate both melted with decomposition at $154\text{--}154.5^\circ$ and an intimate mixture of the two melted at the same temperature. The yield of osazone obtained from the pure aldosemonophosphate was equal to 48 % of the weight theoretically possible. More than half of this yield was obtained from the first period of heating and the melting point of this sample before recrystallisation was identical with that of the recrystallised osazone. The successive products melted within one or two degrees of this temperature.

Analyses. The P content corresponded with that of a phenylhydrazine salt of the osazone of hexosemonophosphoric acid.

Osazone from aldosemonophosphate gave P = 5.63 %.

„ fructosemonophosphate (Neuberg) gave P = 5.61 %.

Calculated for $C_{24}H_{31}O_7N_6P$ = 5.68 %.

The specific rotation of the osazone from aldosemonophosphate was determined in pyridine-alcohol (2:3).

$[\alpha]_{5461}^{21}$ — 60° after 15 min., — 38° after 85 min., — 35° after 24 hrs.

Neuberg and Reinfurth [1924] give $[\alpha]_D - 51^\circ$ after 15 minutes and — 36° after 1 hour (equilibrium) for the phenylhydrazine salt of the osazone of fructosemonophosphate and also for that obtained from hexosediphosphate.

Further evidence was obtained of the identity of the two osazones by measuring the rate of hydrolysis in N H_2SO_4 at 100° . The results are shown below.

	Aldosemono- phosphoric ester	Fructosemono- phosphoric ester %	Osazone of aldosemono- phosphoric ester %	Osazone of fructosemono- phosphoric ester %
Hydrolysis in 1 hour	—	—	47	44
Hydrolysis in 2 hours	0	41	80	75

The two osazones were hydrolysed at approximately equal rates, which were twice as great as that for fructosemonophosphoric ester. The hydrolysis

of aldosemonophosphoric ester was too small to be estimated with the quantities taken.

An osazone was also prepared from the ketose component of the platelet fraction (No. 14) and this also, after recrystallisation, melted at 154° and at the same temperature when mixed with the osazone of fructosemonophosphoric acid.

Hydrolysis of aldosemonophosphoric ester.

Hydrolysis by acids. The hydrolysis of aldosemonophosphoric ester was studied by heating a $0.03M$ solution of the free acid at 100° and also by heating the ester, in similar concentration, with $0.1N$ and with $N H_2SO_4$ at 100° . The results of two experiments are shown in Fig. 1, the actual rates

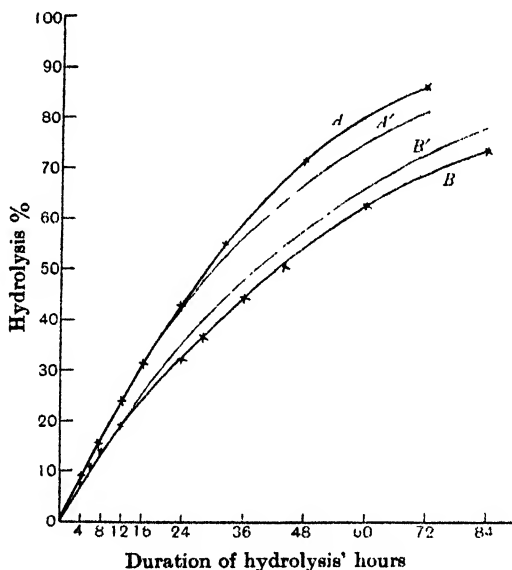


Fig. 1. A. Autolysis of $0.03M$ aldosemonophosphoric ester at 100° .

A'. $k = 0.167 \times 10^{-3}$.

B'. $k = 0.13 \times 10^{-3}$.

B. Hydrolysis of $0.03M$ aldosemonophosphoric ester in $N H_2SO_4$ at 100° .

of hydrolysis being shown by the thick lines while the thin lines are theoretical curves of unimolecular equations drawn for the velocity constants calculated from the results of the first 16 hours' hydrolysis ($k = 1/t \log a/(a - x)$, t being time in minutes). No sharp deviation from these theoretical curves is shown in either case, but a gradual, though slight, falling off in the rate is shown for the hydrolysis in $N H_2SO_4$, while a slight increase is shown in the rate of hydrolysis of the free ester alone. The results appear to support the view that we are dealing with a homogeneous ester. It was very surprising, however, to find that the hydrolysis proceeded more rapidly in the solution containing aldosemonophosphoric acid alone than in presence of $N H_2SO_4$, but this fact was confirmed by a repetition of both experiments. Further, with $0.1N H_2SO_4$

the rate of hydrolysis was very slightly higher (66 % in 60 hours) than with *N* acid (63 % in 60 hours). Is it possible that these results are due to the presence of two ring systems, one of which, favoured by the presence of sulphuric acid, hinders the removal of the phosphoric acid group? The increased specific rotation of the free ester in presence of sulphuric acid might possibly be connected with the formation of this ring and the sensitiveness of the ester towards alkali, noted in reference to the iodimetric estimation, may also be related to such an equilibrium (*cf.* Lohmann's [1928] suggestion quoted on p. 323 of this paper).

The sugars produced from aldosemonophosphoric ester by hydrolysis with 0.1*N* H₂SO₄ and heating the solution of the free acid alone were isolated and examined. The solutions were neutralised with baryta and treated with 4 times their volume of alcohol. The precipitated barium salts were removed by filtration and thoroughly washed with 80 % alcohol. The filtrate was evaporated on the water-bath to a small bulk and then in an evacuated desiccator over sulphuric acid. The results of the examination of the products are shown in Table II. The properties of the sugar obtained from hydrolysis with 0.1*N* H₂SO₄ corresponded very closely with those of glucose. That obtained from the partial hydrolysis of the ester without H₂SO₄ had a somewhat lower specific rotation and was less pure. A control experiment was carried out by heating a solution of glucose for 60 hours with 0.1*N* H₂SO₄ and then recovering the sugar in the manner described above; it is seen from the results that very little decomposition or alteration of the glucose occurred.

Hydrolysis by bone phosphatase. Two experiments were carried out on the hydrolysis of the aldosemonophosphoric ester by the bone phosphatase. 0.4 g. of the pure barium salt was converted into the free acid and the solution (15 cc.) treated with sodium hydroxide to bring the *p*_H to 7.0. A purified preparation of bone phosphatase (50 mg.) was added and the solution kept at room temperature in presence of chloroform, the *p*_H being checked and the liberated inorganic phosphate estimated at intervals. Hydrolysis was practically complete in 4 days. The solution was evaporated to dryness *in vacuo* over sulphuric acid and the residue was extracted repeatedly with hot 80 % ethyl alcohol. The filtrate was evaporated, first on the water-bath and finally in an evacuated desiccator and the residue dissolved in 30 cc. water for examination. The hydrolysis was carried out in neutral solution and at room temperature in order to minimise the risk of the Lobry de Bruyn transformation taking place. The slower rate of hydrolysis at room temperature is compensated for by the fact that no destruction of enzyme occurs (as it does at 37°) so that hydrolysis will continue smoothly even during several weeks. In spite of these mild conditions it was evident that the sugar product contained a considerable proportion of ketose. The analytical results and the preparation of an osazone, which was identified by its melting point (206°) with glucosazone, justified the conclusion that the product contained glucose and fructose. Whether mannose was also present is uncertain, but an attempt to obtain the

phenylhydrazone was not successful. This transformation of the sugar component of monophosphoric ester on hydrolysis with phosphatase is in agreement with the results found by Martland and Robison [1929] for the enzymic hydrolysis of hexosediphosphoric ester. Control experiments carried out in a similar manner with bone phosphatase + distilled water, and with phosphatase + 0.5 % glucose solution showed that only a very small amount of reducing and optically active material could be derived from the phosphatase and that glucose itself suffered no transformation under the conditions of the hydrolysis.

Table II.

Method of hydrolysis	Duration of hydrolysis hrs	Hydrolysis %	Wt. of hexose equivalent to P liberated mg.	Wt. of syrup dried over H_2SO_4 mg.	Reducing power as glucose (mg.)		Fructose (Seli-vanoff) mg.	$[\alpha]_{5461}^{20}$ calculated on the H. and J. value
					H. and J.	Iodine		
1. 0.1 N H_2SO_4 at 100°	60	66	86	83	62	64	6	+ 60.5°
2. Free acid alone at 100°	44	73	92	118	88	92	7	+ 54°
3. Bone phosphatase at room temperature and p_H 7.0	96	99	144	155	133	116	24	+ 33°
4. Bone phosphatase at room temperature and p_H 7.0 (Results of 3 and 4 corrected for phosphatase blank)	80	97	82	—	82	67	13	+ 29°
Controls:				Wt of glucose taken				
20 cc. 0.5 % glucose solution + 80 cc. alcohol evaporated to dryness and extracted with 80 % alcohol				100	100	96	97	1 - 63°
27 cc. 0.5 % glucose solution heated with 0.1 N H_2SO_4 60 hrs.; neutralised and treated as above				135	155	125	133	6 - 62°
30 cc. 0.5 % glucose solution + 50 mg. bone phosphatase evaporated to dryness in desiccator and extracted with 80 % alcohol. (Results corrected for phosphatase blank)				150	160	143	147	2 + 63°
30 cc. H_2O + 50 mg. bone phosphatase evaporated to dryness in desiccator and extracted with 80 % alcohol				0	5	0.4	1.3	0 $\alpha_{5461} = -0.04^\circ$ ($l = 4$ dm.)

Preparation of a phosphohexonic acid from hexosemonophosphoric ester.

A preparation of hexosemonophosphate, which had been purified by recrystallisation of the brucine salt from 20 % ethyl alcohol and contained about 80 % of the aldose ester, was oxidised with bromine in order to convert this ester into the corresponding phosphohexonic acid. To 10 g. of the barium salt, dissolved in 80 cc. water, 1.4 cc. bromine and 12 g. barium carbonate were added, a further 1.4 cc. bromine being added after 24 hours. The solution was kept at room temperature and shaken at intervals. Iodimetric estimations carried out on 0.2 cc. portions, after removal of the bromine, indicated that oxidation was complete after 48 hours. The excess of bromine was removed by aeration and the barium carbonate by filtration. The filtrate (p_H 3.5) was poured into 5 times its volume of alcohol upon which the acid salt of the phosphohexonate was precipitated. This was filtered off, washed thoroughly with 85 % alcohol and dried. It was then dissolved in 20 cc. water with the aid of 2 cc. N HCl and, after filtration, treated with a solution of barium

hydroxide till pink to phenolphthalein. The precipitate was filtered off, washed with water and absolute alcohol and dried over sulphuric acid *in vacuo*. It was non-reducing and gave a negative Selivanoff reaction.

P found 6.42 %; calculated for $(C_6H_{10}O_{10}P)_2Ba_3$, P = 6.48 %.

$[\alpha]_{5461}^{21} = -1.5^\circ$.

Solubility in water at 100°. 100 cc. solution contained 0.71 g.

The acid barium salt was prepared by dissolving 1 g. of the neutral salt in sufficient dilute hydrochloric acid to make the solution just acid to methyl orange and adding 5 times the volume of alcohol. The precipitate was filtered off, washed thoroughly with 85 % and absolute alcohol and dried *in vacuo*.

P found 7.40 %; calculated for $C_6H_{11}O_{10}P Ba$, P = 7.54 %.

$[\alpha]_{5461}^{21} = +0.2^\circ$.

A solution of the free phosphohexonic acid was prepared by decomposing 0.25 g. of the barium salt with the exact amount of sulphuric acid and removing the barium sulphate by centrifuging. The change in rotation due to lactone formation is shown below, the times being measured from the addition of the sulphuric acid. After heating to 70° the solution was rapidly cooled.

Time	α_{5461}^{21} ($l = 4$ dm.)	$[\alpha]_{5461}^{21}$
10 minutes at 21°	0	0
2 hours at 21°	+0.03°	+2°
18 hours at 21°	+0.08°	+5°
1 hour at 70°	+0.26°	+16°
2 hours at 70°	+0.285°	+18°

The solution was finally heated for an hour at 70° with 0.1N HCl and cooled rapidly when the $[\alpha]_{5461}$ was +21°. The yield of phosphohexonate was equivalent to 62 % of the original hexosephosphate, but a further quantity of less pure salt was recovered from the first acid-alcohol filtrate together with a small amount of fructosemonophosphate.

*Preparation of hexonic acid by hydrolysis of the phosphohexonic acid
with bone phosphatase.*

The hydrolysis of the phosphohexonate was carried out by means of bone phosphatase in two separate experiments.

1st method. 1 g. of the neutral barium salt in 20 cc. water was treated with 0.5 g. of purified phosphatase at 37° and p_H 8.6–8.8. The flask was shaken at frequent intervals and barium hydroxide solution added as required to maintain the stated p_H . Hydrolysis was complete in about 9 hours and the solution was then heated at 100° and filtered. The residue was well washed with hot water and the filtrate and washings were concentrated on the water-bath. The barium hexonate was converted into the calcium salt by treatment with a solution of calcium sulphate. The filtered solution was again concentrated and poured into absolute alcohol to precipitate the salt, which was then filtered off, washed with alcohol and dried *in vacuo*. The salt was free from phosphate but estimation of the calcium indicated that it contained a little calcium sulphate which could not be easily removed.

2nd method. 1.35 g. barium phosphohexonate was treated with the exact quantity of sulphuric acid required to precipitate the barium and the sulphate removed by centrifuging and thoroughly washed. The acid solution was heated with pure calcium carbonate, filtered and treated with sufficient calcium hydroxide to raise the p_H to 6.0. (The addition of more calcium hydroxide caused the salt to be precipitated.) After cooling the solution was treated with 0.5 g. of purified bone phosphatase and a few drops of chloroform and was left in a stoppered flask at room temperature. Each day the p_H was noted but required no adjustment and the progress of the hydrolysis was determined by estimating the inorganic phosphate in 0.2 cc. Hydrolysis was complete in 4 days, after which the solution was heated on the water-bath and treated with calcium hydroxide until pink to phenolphthalein. It was filtered and the residue washed with boiling water. The filtrate was concentrated on the water-bath and poured into absolute alcohol; the precipitate was filtered, washed with alcohol, dried, and purified by solution in water and reprecipitation with alcohol. The salt was free from phosphorus and did not reduce hypiodite. By the Hagedorn and Jensen method a very slight reduction (1 %) was found.

Ca found 9.4 %; calculated for $(C_6H_{11}O_7)_2Ca$, 9.30 %.

$[\alpha]_{5461}^{20} + 6.8^\circ$ ($l = 4$ dm., $c = 0.8$ %).

The recorded values for the $[\alpha]_D$ of calcium gluconate vary between $+6^\circ$ and $+10.5^\circ$.

A solution of the free acid was produced by treating the calcium salt with the calculated quantity of oxalic acid and filtering. The change in rotation due to lactone formation is shown below.

Time	α_{5461}^{20} ($l = 4$ dm.)	$[\alpha]_{5461}^{20}$
20 minutes at room temperature	-0.08°	-2.9°
18 hours at room temperature	$+0.22^\circ$	$+8.6^\circ$
2 hours at 70°	$+0.54^\circ$	$+21.0^\circ$
3 hours at 70°	$+0.59^\circ$	$+22.9^\circ$
In 0.1N HCl 2 hours at 70°	$+0.71^\circ$	$+27.8^\circ$
3 hours at 70°	$+0.71^\circ$	$+27.8^\circ$
66 hours at room temperature	$+0.52^\circ$	$+20.4^\circ$

The results agree with the recorded values for d -gluconic acid if the factor 1.18 is used to convert $[\alpha]_D$ into $[\alpha]_{5461}$. Rehorst [1928] gives the following values for $[\alpha]_D^{20}$ in 2.8 % aqueous solution: after 5 mins. -6.72° ; after 20 mins. -2.75° ; after 24 hrs. $+7.02^\circ$; after 5 days $+11.9^\circ$. In Tollen's "Handbuch der Kohlenhydrate" (3rd edition) the equilibrium mixture obtained on heating the free acid is stated to have $[\alpha]_D +23.4^\circ$, which, multiplied by 1.18 gives $[\alpha]_{5461} +27.6^\circ$.

Oxidation of aldosemonophosphoric acid with nitric acid.

It was considered that if the phosphoric acid group is in position 3 of the aldose molecule, or in any position other than 6, it should be possible to obtain

a phosphodicarboxylic acid by oxidation of the ester itself or of the phosphohexonate with nitric acid. Accordingly, a number of attempts to prepare such a compound were made by treating the pure aldosemonophosphate and phosphohexonate described above with nitric acid of different concentrations, at different temperatures, and for various periods of time. The phosphohexonate manifested a considerable resistance to further oxidation under mild conditions and when oxidation did occur it was invariably accompanied by hydrolysis. The products obtained from this and from the aldose ester were always mixtures containing dicarboxylic acids, but in no case could the analytical results be interpreted as providing evidence of the presence of a dicarboxylic acid in which the phosphoric group was still present.

DISCUSSION.

The results we have described lead us to conclude that one, and possibly the chief, constituent of hexosemonophosphoric ester of fermentation has been isolated in pure homogeneous condition and that this compound has the constitution of an aldosemonophosphoric ester. The properties of the sugar obtained from this ester by acid hydrolysis as well as those of the hexonic acid formed from it by oxidation with bromine followed by enzymic removal of the phosphate group, provide evidence of the identity of the aldose with *d*-glucose. Hydrolysis of the ester by phosphatase has been shown to involve partial transformation of the hexose as already demonstrated for the hydrolysis of fructosediphosphate. This transformation is in itself of interest but the properties of the sugar produced obviously cannot be accepted as proving the nature of the aldose present in the ester.

For the position of the phosphoric acid group in this ester 2 is excluded by the formation of a phosphohexosazone, while the formation of both stable and unstable methylhexosides [King and Morgan, 1929; King, McLaughlin and Morgan, 1931] very probably excludes positions 4 and 5. Position 6 appeared at first to be excluded by the differences between the melting points of the osazone originally obtained from hexosemonophosphoric ester and of the osazone prepared by Young from fructosediphosphoric ester and later by Neuberg and Reinfurth [1924] from fructosemonophosphoric ester. This difficulty has disappeared since the osazone now prepared from the pure aldosemonophosphate has been found to have the same melting point and rate of hydrolysis as the osazone of fructosemonophosphoric ester in which the phosphoric acid group is considered to occupy position 6.

Our failure to obtain a phosphodicarboxylic acid by oxidation of the aldosemonophosphoric ester with nitric acid further supports the view that the ester is a glucose-6-phosphate. The properties of the pure ester do not, however, entirely agree with those of any of the synthetic glucosephosphates which have been prepared by a number of workers [Komatsu and Nodzu, 1924; Nodzu, 1926; Raymond and Levene, 1929; Josephson and Proffe, 1930; Levene and Raymond, 1930]. In their last paper Levene and Raymond [1930]

report a new examination of the ester synthesised from di-*isopropylidene*-glucose and conclude, in agreement with Josephson and Proffe, that this ester is glucose-3-phosphate, and that it differs from the hexosemonophosphate of fermentation in its specific rotation and its reaction with phenylhydrazine, with which it forms a 3:6-anhydrohexosazone. They also find that its rate of fermentation is much slower than that of the natural ester. Levene and Raymond have further synthesised a phosphoric ester from *isopropylidene*-glucose which they consider to be glucose-6-phosphate and have shown that it gives a phosphohexosazone identical with that obtained from the Harden and Young and Neuberg esters, and that its rate of fermentation is identical with that of hexosemonophosphate. The specific rotations given for this synthetic ester and its barium salt do not, however, agree with those of the pure aldosemonophosphoric ester described in the present communication.

By multiplying Levene and Raymond's values for $[\alpha]_D$ of their synthetic esters by the factor 1.18 the following values for $[\alpha]_{5461}$ are obtained for comparison with those of the aldose ester here described:

	$[\alpha]_{5461}$ of barium salt	$[\alpha]_{5461}$ of free acid
Synthetic ester from di- <i>isopropylidene</i> glucose (glucose-3-phosphate)	+31.3°	+46.6°
Synthetic ester from <i>isopropylidene</i> glucose (glucose-6-phosphate)	+15.3°	+30.6°
Pure aldosemonophosphoric ester	+21.2°	+41.4°

Although Levene and Raymond state that the ester prepared from mono-acetone glucose may not have been quite pure, the differences in the specific rotations are sufficiently great to cause hesitation in concluding that this synthetic ester is identical with the aldosemonophosphate of fermentation.

Apart from this discrepancy all the evidence at present available goes to show that the natural ester whose isolation and properties we have described is glucose-6-phosphate. From the iodimetric estimations it would appear that this ester cannot normally form more than about 50–70 % of the hexosemonophosphate isolated from the products of fermentation, while it may well be that part of this percentage represents other esters, *e.g.* a disaccharide-diphosphate, which would account for the low Hagedorn and Jensen reducing power of some of the other fractions isolated during the investigation of the mixed hexosemonophosphate. That the latter contains also the Neuberg fructosemonophosphate seems to be certain, but whether this ester accounts for the whole of the non-aldose fraction, as estimated iodimetrically, will not be settled until the properties of the unknown ester are determined.

Trehalosemonophosphoric ester has not been isolated from these yeast juice products, although the high specific rotation of certain small fractions obtained in the crystallisation of the brucine salt might be accounted for by its presence. Monophosphoric esters (not yet obtained in pure condition) of higher specific rotation than that of aldosemonophosphate have been described by Euler, Myrbäck and Runejelm [1928] and by I. S. Neuberg

and Ostendorf [1930] (from the products of fermentation of mannose by fresh yeast and toluene), but in the case of these compounds also the presence of trehalosemonophosphate in small proportion might possibly account for the high specific rotation recorded by the authors. It does not, however, necessarily follow that this is the true explanation and further information with regard to these esters will be awaited with interest.

SUMMARY.

1. An aldosemonophosphoric ester has been isolated in pure condition from the hexosemonophosphoric ester produced by fermentation of hexoses with yeast juice.
2. The $[\alpha]_{546}^{20}$ of the free ester is $+41.4^\circ$, and of the barium salt $+21.2^\circ$ ($c = 8.4\%$). The reducing power of the ester by an iodimetric method is equal to that of the equivalent amount of glucose. Its reducing power by the Hagedorn and Jensen method is 80% of that of glucose. The Selivanoff reaction is not appreciably greater than that given by glucose.
3. The phenylhydrazine salt of the osazone of aldosemonophosphoric ester has been prepared and shown to be identical both by its melting point and its rate of hydrolysis with the corresponding salt of the osazone of fructosemonophosphoric ester; m.p. $154-154.5^\circ$.
4. The ester is very resistant to hydrolysis by acids at 100° . Hydrolysis proceeds more rapidly when the free aldosemonophosphoric acid is heated alone than in presence of sulphuric acid. The sugar product of acid hydrolysis has the character of *d*-glucose.
5. Hydrolysis by bone phosphatase proceeds rapidly at room temperature and p_H 7.0, but the sugar product contains both glucose and fructose.
6. Oxidation of the aldosemonophosphoric ester with bromine yields a phosphohexonic acid from which gluconic acid has been obtained by hydrolysis with bone phosphatase.
7. Oxidation with nitric acid failed to yield any evidence of the production of a dicarboxylic acid containing a phosphoric acid group.
8. These experiments in conjunction with those reported by King, McLaughlin and Morgan point to the constitution of the ester being that of a glucose-6-phosphate.
9. The specific rotation of the free ester and of its barium salt differ considerably from those of the synthetic ester prepared by Levene and Raymond from isopropylideneglucose, and considered by them to be glucose-6-phosphate.
10. A ketosemonophosphoric ester similar in most respects to Neuberg's fructosemonophosphoric ester has also been isolated from the fermentation products, while evidence has been obtained of the presence of another ester as yet unidentified.

REFERENCES.

- Euler, Myrbäck and Runehjelm (1928). *Arkiv Kemi Min. Geol.* **9**, 1.
Harden and Robison (1914). *Proc. Chem. Soc.* **30**, 16.
Josephson and Proffe (1930). *Liebig's Ann.* **481**, 91.
King, McLaughlin and Morgan (1931). *Biochem. J.* **25**, 310.
—— and Morgan (1929). *Chem. Ind.* **48**, 143.
Komatsu and Nodzu (1924). *Mem. Coll. Sci., Kyoto*, **7**, 377.
Levene and Raymond (1930). *J. Biol. Chem.* **89**, 479.
Lohmann (1928). *Biochem. Z.* **194**, 306.
Macleod and Robison (1929). *Biochem. J.* **23**, 517.
Martland and Robison (1929). *Biochem. J.* **23**, 237.
Meyerhof and Lohmann (1927). *Biochem. Z.* **185**, 113.
Neuberg, I. S. and Ostendorf (1930). *Biochem. Z.* **221**, 154.
Neuberg and Reinfurth (1924). *Biochem. Z.* **146**, 589.
Nodzu (1926). *J. Biochem. (Japan)*, **6**, 31.
Raymond and Levene (1929). *J. Biol. Chem.* **83**, 619.
Rehorst (1928). *Ber. deutsch. chem. Ges.* **61**, 163.
Robison (1922). *Biochem. J.* **16**, 809.
—— and King (1929). *Chem. Ind.* **48**, 143.
—— and Morgan (1930). *Biochem. J.* **24**, 119.
Sobotka (1926). *J. Biol. Chem.* **69**, 267.
Willstätter and Schudel (1918). *Ber. deutsch. chem. Ges.* **51**, 780.
Young (1911). *Biochem. Z.* **32**, 178.

XLI. STUDIES IN BLOOD GLYCOLYSIS.

PRELIMINARY OBSERVATIONS.

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THIS study was undertaken to determine the factors concerned in glycolysis in blood incubated at 37°. It is clear from the work of a number of observers—Schmitz and Glover [1927], Stammers [1926] and Chahovitch [1926]—that (1) sterile blood loses its sugar if kept at room or body temperature; (2) the rate of glycolysis varies in different species.

Glycolysis was studied under the following conditions, viz. (1) in whole blood after varying periods of starvation and after feeding, (2) in laked corpuscles, (3) in serum, (4) in cyanide blood, (5) in washed erythrocytes, (6) in washed erythrocytes and glucose solution.

Method.

Our observations have been confined mainly to dog's blood. About 2 cc. of blood were drawn from one of the leg veins and incubated at 37° for a variable period; samples were taken therefrom before incubation and at intervals of 30, 60 and 120 minutes for sugar estimation by MacLean's method. During the process of taking blood asepsis was observed although it was found that this precaution was not necessary at any rate for incubation periods not extending beyond 3 hours.

It will be seen on reference to Table I that the rate of glycolysis is greatest during the first hourly period of incubation; it is greater in blood samples taken about 2 hours after the ingestion of food than after a day's starvation and it decreases considerably after 2 days' starvation.

Effect of cyanide.

To determine whether glycolysis in blood is largely or entirely an oxidative process, blood was mixed with different quantities of cyanide solution in normal saline as follows: 2 cc. blood samples were taken and mixed with 1 cc., 0.5 cc. and 0.2 cc. of 0.3 % KCN solution and the total volume was made up to 3 cc. with normal saline. A control containing 2 cc. blood and 1 cc. saline was also set up. Preliminary glucose estimations were made and the samples were incubated at 37° in small test-tubes. It will be noticed

(Table II) that the quantity of cyanide used has no effect on glycolysis in whole blood and there is no relationship between the quantity of cyanide used and the degree of glycolysis.

Table I.

Blood-sugar in mg./100 cc.	Rate of glycolysis in mg. glucose per 100 cc.		
	$\frac{1}{2}$ hour	1 hour	2 hours
(a) Two hours after food:			
108.5	19	28	48.5
104.5	19.5	42.5	53.5
97	5	24	35
111	9	22	37
Average	13.1	29	43.5
(b) Starvation (1 day):			
87	13	24	39
105.5	8.5	25	31.5
101	10	22.5	38
102	—	—	33
Average	10.5	23.8	35.4
(c) Starvation (2 days):			
71.5	4	11	13.5
73	13	15	20
90.5	2.5	8	25
Average	6.5	11.3	19.5

Table II.

	Composition of incubated blood sample			Blood- sugar in mg./100 cc.	Rate of glycolysis in mg. glucose per 100 cc.	
	Blood cc.	KCN cc.	Saline cc.		1 hour	24 hours
(1)	2	1	—	82	10	34
(2)	2	0.5	0.5	82	10	22
(3)	2	0.2	0.8	82	8	22
(4)	2	—	1	60	—	30

Table III.

Composition of incubated sample		Sugar in mg./100 cc.	Rate of glycolysis in mg. glucose per 100 cc.	
			1 hour	24 hours
(a) Serum		114	2	—
		113	5.5	—
		115	4	—
		111	6	—
		Average	4.4	
(b) 2 cc. serum + 1 cc. erythrocytes		104	20	—
(c) 2 cc. glucose solution in saline + 1 cc. erythrocytes		172	8	65
(d) 2 cc. glucose solution in dist. water + 1 cc. erythrocytes (laked corpuscles)		172	6.5	13

Observations were then made on glycolysis in serum, in laked blood and in glucose solutions in the presence of intact washed erythrocytes, to note the rate of glycolysis and the part played by the corpuscles, if any, in the pheno-

menon. Glycolysis was found to occur to a slight degree in the course of 1 hour in incubated serum and in laked corpuscles in contrast to the findings of Katyama [1926] (Table III, *a*, *d*) and to be greater in serum or glucose solutions in the presence of intact washed erythrocytes (Table III, *b*, *c*).

The results obtained above in (*c*) and (*d*) are not affected by the substitution of glucose dissolved in serum for an ordinary glucose solution.

DISCUSSION.

In connection with the increased glycolysis after the ingestion of food as compared with the amount of glycolysis in the blood from a starving animal, three possible factors at least must be taken into consideration.

- (1) Increased percentage of sugar in the blood.
- (2) Increased amount of glycolytic substance in the blood.
- (3) Effect of increased amount of insulin released (following the ingestion of food) on the tissue cells leading to an increased liberation of the substance concerned with glycolysis.

Observations on these points are being continued.

Further, the question arises as to the factors concerned in glycolysis and the nature of the process of glycolysis. It might be supposed that the latter is one of oxidation but our observations confirm those of Abraham and Altmann [1927] in that cyanide, which inhibits oxidising enzymes, has no effect on the glycolytic power of blood.

From the result that glycolysis proceeds most rapidly in whole blood as compared with the rate of glycolysis in serum, glucose solutions + intact erythrocytes and glucose solutions + laked erythrocytes, it may be inferred that there are factors present in both serum and erythrocytes which are essential for the increased glycolysis observed. Further work is being done to elucidate this point.

SUMMARY.

1. Starvation decreases and the administration of food increases the rate of glycolysis in blood.
2. The factors responsible for glycolysis in blood are present in both erythrocytes and serum.

REFERENCES.

- Abraham and Altmann (1927). *Klin. Woch.* **6**, 456.
Chahovitch (1926). *Compt. Rend. Soc. Biol.* **94**, 691.
Katyama (1926). *J. Lab. Clin. Med.* **12**, 239.
Schmitz and Glover (1927). *J. Biol. Chem.* **74**, 761.
Stammers (1926). *Trans. Roy. Soc. S. Africa*, **13**, 323.

XLII. THE ADDITION COMPOUND THEORY OF ENZYME ACTION.

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(Received December 24th, 1930.)

1. INTRODUCTION.

IN 1929 the writer, as a result of a study of three enzymes in *B. coli communis* which act on fumaric acid, propounded "the rather attractive hypothesis that one of the conditions for a reaction to occur at these enzymes is that all the substrates shall be combined there together—succinic acid and methylene blue for succinoxidase action, fumaric acid and ammonia with aspartase, and fumaric acid and water with fumarase" [Woolf, 1929]. Since that time, further experimental findings, coupled with a thorough study of the work of others, have led the writer to develop this hypothesis into a definite theory of enzyme action, which it is the object of this paper to describe.

According to the view about to be put forward, the meaning of the term substrate is widened to include all the molecules participating in the catalysed reaction, including, for example, water in a hydrolysis, and the methylene blue or other hydrogen acceptor in a dehydrogenase action. The addition compound theory may then be stated as follows. An enzyme is a definite chemical compound which is able to form an unstable addition compound with all its substrates, each at its own specific combining group in the enzyme molecule. The process of catalysis then consists of a series of tautomeric changes in the enzyme-substrate complex, as a result of which, in a certain proportion of cases, the complex is able to dissociate into free enzyme plus the products of the catalysed reaction.

The line of reasoning on which this theory is based falls naturally into two sections, first an examination of all the known data of enzyme kinetics, and the induction from them of general laws, followed by the deduction from these laws of the probable mechanism of the catalytic process. A complete presentation of the first section is obviously impossible in this place. The full evidence for the conclusions given below will be set out, however, in a review by the writer on "Enzyme Kinetics," which is now in course of preparation for *Biological Reviews*.

2. ENZYME KINETICS.

The enzyme-substrate compound.

The hypothesis that the enzyme and substrate unite stoichiometrically was first put forward explicitly by Michaelis and Menten [1913], who deduced from it the well-known equation

$$V = \frac{U \cdot [E] \cdot [S]}{K + [S]},$$

where $[E]$ and $[S]$ represent the concentrations of enzyme and substrate, V the velocity of the catalysed reaction, U the maximum velocity for unit enzyme concentration, and K , the "Michaelis constant," the concentration of the substrate at which half the maximum velocity is attained. The reciprocal of K is the apparent affinity of the enzyme for the substrate.

This equation was found by Michaelis and Menten to hold with great accuracy for the action of saccharase on sucrose, and has since been found to fit the results obtained with many other enzymes. The full accounts of the many applications of this equation which are given by Kuhn [1925] and Haldane [1930] leave no doubt that the hypothesis of a stoichiometric enzyme-substrate compound is in good accord with the facts of enzyme kinetics.

The p_H -activity curve.

Many enzymes give p_H -activity curves showing an optimum p_H about which the curve is more or less symmetrical. Several authors have interpreted these curves as being due to the ionisation of the enzyme as an ampholyte, the isoelectric or un-ionised form of the enzyme alone being catalytically active, activity being determined not by the charge on the colloidal enzyme complex as a whole, but solely by the state of ionisation of one definite acidic and one basic group in the enzyme molecule. This view is supported by a considerable body of evidence, which is of two types: that based upon the shape of the curve, and that drawn from the effects of salts on the reaction rate.

Quantitative deductions from the shape of the curve can only be made when it covers eight or more p_H units. On the ampholyte theory, the curve would fall away from the optimum in a Henderson-Hasselbalch curve if the affinity did not change with p_H . If the affinity falls as the p_H recedes from the optimum, the curve will be steeper than a true dissociation curve, to an extent depending on the change in affinity with the p_H . These theoretical predictions have been realised, for instance by Josephson [1925] for β -glucosidase, and for saccharase, which is discussed by Kuhn [1925] and Haldane [1930]. Evidence from the effects of salts is given in such studies as those of Myrbäck [1926, 1] on saccharase and [1926, 2] amylase, and by Mann and Woolf [1930] on fumarase. These authors found that salts affected the velocity of the catalysed reaction in just the way that would be expected if the salt, or one of its ions, combined with the enzyme and altered the p_K value of one

or both of the critical ionisable groups, and hence the proportion of enzyme molecules in the isoelectric or active form at any given p_H .

Now there are many enzymes which do not give a p_H -activity curve of the symmetrical type, as for example the dehydrogenases [Dixon and Thurlow, 1924; Quastel and Whetham, 1924; and unpublished observations in this laboratory] and catalase [Michaelis and Pechstein, 1913]. Though it does not seem to have been pointed out before, the ampholyte type of curve is not characteristic of all enzymes, but only of those of a particular class—the enzymes which catalyse reactions in which water is one of the substrates. The non-hydrating enzymes mentioned above give a curve which rises in the acid range until a maximum velocity is reached, which persists unchanged until the enzyme begins to be destroyed by excessive alkalinity. Such a curve suggests that the enzyme acts as an ionised acid or an un-ionised base, and this suggestion is borne out by the fact that Michaelis and Pechstein [1913] found that the p_H -activity curve of liver catalase is a very accurate Henderson-Hasselbalch curve, which is shifted by salts in a manner analogous to the p_K -shifts found for the hydrating enzymes.

The enzyme a chemical compound.

All the kinetic results indicated above suggest strongly that an enzyme is a definite chemical structure. Further reasons for this conclusion are given by Willstätter [1927] in a summary of his work on enzyme purification, and by Haldane [1930] on several other grounds.

3. THE ADDITION COMPOUND THEORY.

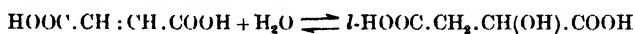
The activation of the substrate.

It is obvious that the substrate must be distorted or activated in some way at the enzyme in order that reaction may occur, and various mechanisms, such as the electric field of Quastel [1926], have been proposed to account for this activation. All such theories are compelled to assume a double mechanism at the enzyme, the activating mechanism and some chemical grouping which will bind the substrate while it is being activated and undergoing reaction [cf. Quastel and Wooldridge, 1927, 1, 2]. It seems to the writer that this double mechanism is an unnecessary assumption. When the substrate is bound at the enzyme, becoming part of a temporary enzyme-substrate complex, the very chemical forces which bind it must surely produce sufficient distortion in its molecule to make it abnormally reactive. Until the chemical nature of enzymes is discovered, the nature of these forces can only be the subject of more or less plausible speculation. It seems likely, however, that since substrates are often stable, saturated molecules—e.g. succinic acid—the union with the enzyme is of the nature of a multiple co-ordination compound or chelate ring, which, as shown by Sidgwick [1927], involves the polarisation of the molecules which are so linked. But whether this or some other

mechanism is the true one, it seems to the writer an economy of hypotheses to assume that the substrate-binding and substrate-activating processes are one and the same until evidence to the contrary is produced. This view is also put forward in a slightly different form by Haldane [1930]. It may be assumed, then, that when the enzyme and substrate are combined, the chemical structures of the molecules are so altered that the complex will readily undergo further tautomeric changes, which constitute the actual catalytic process. The nature of these changes is discussed below.

The mode of action of a hydrating enzyme.

A convenient illustrative enzyme is fumarase [*cf.* Woolf, 1929], which catalyses the reversible reaction



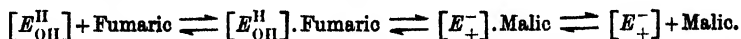
since the complication is absent of the hydrated product splitting into two molecules, and the forward and reverse reactions can be readily induced, so that the mechanism of both can be discussed. The kinetic results already considered show that the conversion of fumaric to malic acid can only occur at an enzyme molecule which is combined with fumaric acid and in the isoelectric form. It may therefore be assumed that for reaction to be effected there must be present at the enzyme a fumaric acid molecule, a hydrogen ion, and a hydroxyl ion. Now the reaction product, malic acid, is formed by the addition of these three bodies. It is therefore a natural step to assume that the enzyme can only act in the fully hydrated form because the hydrogen and hydroxyl ions are substrates for the reaction, as well as the fumaric acid.

An enzyme molecule can be pictured uniting with a molecule of fumaric acid. In the majority of cases, the substrate molecule will dissociate off unchanged. But while the substrate is at the enzyme, its structure is distorted, it is polarised by the chemical forces holding it there, in such a way that it is able to "accept" a hydrogen and a hydroxyl ion at the two carbon atoms of the structure —CH:CH— . If these two ions are also present at the enzyme at the same time, in a given proportion of cases the enzyme-fumaric-water addition compound will tautomerise so that the water ions leave the enzyme and add themselves across the double bond of the substrate, which will thus become free to dissociate off as malic acid. When this occurs, it is important to notice that the enzyme is left with neither a hydrogen nor a hydroxyl ion, *i.e.* in the zwitterion form.

There are thus four forms of the enzyme to consider—the dissociated acid, the dissociated base, the fully hydrated, and the zwitterion form. The last two together constitute the isoelectric form of the enzyme, which will be partitioned into hydrated and zwitterion forms in a constant ratio, which is independent of the p_{H} .

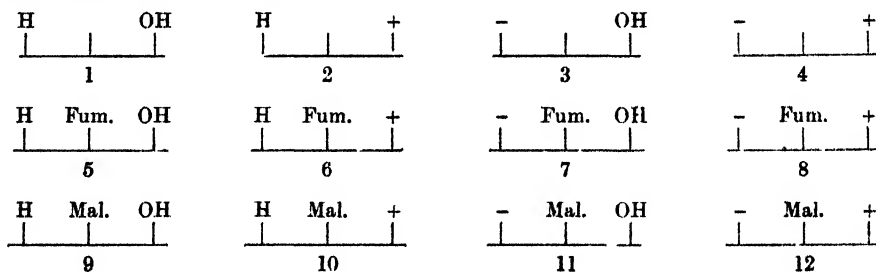
Consider now the reverse reaction. When a molecule of malic acid combines at the enzyme, it too will undergo polarisation so that the elements of

water become dissociable as ions. If the enzyme already has a hydrogen or a hydroxyl ion, it will be unable to take up water from the substrate, and reaction cannot occur. If however it is in the zwitterion form, in a proportion of cases water will leave the substrate and combine with the enzyme, the substrate then dissociating off as fumaric acid and leaving the enzyme fully hydrated. The equilibrium can therefore be represented



The mechanism here suggested thus conforms to the thermodynamic requirement that the reverse reaction in a reversible process shall pass through exactly the same stages, in the opposite order, as the forward process. The essential features of the process may be expressed as follows. The fumaroid skeleton, when combined with the enzyme, is so polarised that it acquires temporary acidic properties at one carbon atom and basic properties at the other. The complex thus becomes a dibasic acid and diacidic base, and the hydrogen and hydroxyl ions will partition themselves, statistically, between the acidic and basic groups of the enzyme and the substrate in a proportion determined by the relative affinity of the enzyme and the substrate for the water. It is obvious that from such a process a true thermodynamic equilibrium will be obtained.

In a system containing fumarase, fumaric acid and water there are the following forms of the enzyme to be considered:



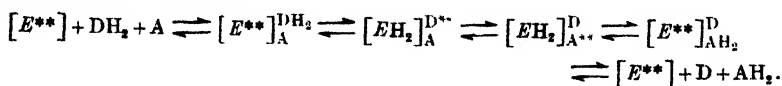
Form 5 is catalytically active in the forward reaction and form 12 in the reverse direction. All these forms are in equilibrium with one another and with fumaric acid, malic acid and the ions of water in the medium, and the affinity of each ionic species of the enzyme for fumaric acid and malic acid will, in general, not be the same. The state of affairs is therefore rather complex. It is however amenable to treatment by the law of mass action, and is then found to lead quantitatively to the kinetic results which are actually found by experiment, and which are set out in Section 2 of this paper.

The above line of argument applies to any hydrating enzyme, with the complication that when the catalysed reaction is a hydrolysis, the reverse reaction involves two substrates, each of which must combine with the enzyme, with its own characteristic affinity.

The mode of action of a dehydrogenase.

Succinoxidase will be taken as an example, since it is the only dehydrogenase whose thermodynamic reversibility has been proved experimentally, and methylene blue will be considered to be the hydrogen acceptor. The catalysed reaction then consists of the transfer of two hydrogen atoms from the donator to the acceptor. Many considerations render it probable, however, that the actual act of oxidation consists in the transfer of two electrons to the dye from the donator, which has previously lost two hydrogen ions, the process being completed by the acquisition of two hydrogen ions by the charged acceptor. One must therefore visualise the donator being polarised by its combination with the enzyme so as to acquire acidic properties at the structure $-\text{CH}_2.\text{CH}_2-$, the enzyme, in a given proportion of cases, taking up the two temporarily ionisable hydrogens from the donator in the same way as fumarase takes up the temporarily ionisable hydrogen and hydroxyl from malic acid. In order to be able to do this, the enzyme must itself be an ionised acid, which is precisely the conclusion that is indicated by the shape of the p_{H} -activity curve.

There is good kinetic evidence that the donator must combine with the enzyme. Reasons were given by Woolf [1929] for supposing that the acceptor must also combine. These reasons are reinforced by the work of Bertho [1929], who found that quinone, acting as hydrogen acceptor with the dehydrogenases of certain lactic acid bacteria, gave a good Michaelis curve. Unpublished observations by the writer show that the same is true for methylene blue and other dyes acting as hydrogen acceptors with a variety of dehydrogenases. In order to be catalytically active, therefore, a dehydrogenase molecule must have combined at it a molecule of donator and a molecule of acceptor, and be itself dissociated as an acid. If DH_2 and D represent the reduced and oxidised forms of the donator, AH_2 and A the corresponding forms of the acceptor, E the enzyme and $*$ an electron, the sequence of changes may then be formulated as follows:



In this scheme the enzyme is represented as a dibasic acid, but the same general result would be obtained if it were taken as monobasic, though the formulation would be a little more lengthy. The doubly charged enzyme combines with both substrates. The donator is activated, and parts with two hydrogen ions to the enzyme. The two excess electrons are then transferred from the donator to the acceptor, this being the actual act of oxidation. The acceptor then acquires two hydrogen ions, and both substrates dissociate off in the altered condition, leaving the enzyme in its initial state. Since all the intermediate forms are in dynamic equilibrium, it is probable that only a small proportion of the enzyme-substrate complexes formed will be completely

transformed, and most will dissociate into enzyme and unchanged substrates. When the sequence of changes is read in the opposite order, it represents the reverse reaction—*e.g.* the reduction of fumaric to succinic acid by leuco-methylene blue. This mechanism, like that suggested for the hydrating enzymes, is therefore thermodynamically sound.

Similar considerations could be applied to other types of enzyme action, but these two examples suffice to indicate the scope and method of the theory. While it is claimed that the central idea is new, the writer wishes to acknowledge that he has freely drawn on the hypotheses of many previous workers, and adopted from them whatever elements seemed to him of value. It is claimed for this theory that it contradicts no known facts; that it gives a unitary explanation of the phenomena of enzyme kinetics, including p_H -activity curves, which no previous theory has professed to explain; that the mechanism of enzyme action proposed is a plausible one; and that it is a useful working hypothesis, in that it enables many quantitative predictions to be made, which can be tested by clear-cut experiments. It has certainly proved of extraordinary value as a working guide to the writer, and it is hoped to publish in the near future several enzyme studies suggested by the theory. The writer hopes that it may also prove of value to other workers in the field of enzyme action.

SUMMARY.

The theory is proposed that an enzyme is a definite chemical compound, which is able to form an addition compound with all its substrates, the actual process of catalysis consisting of a series of tautomeric changes in this enzyme-substrate complex, as a result of which, in a certain proportion of cases, it is able to dissociate into free enzyme and reaction products. This theory is shown to account in a quantitative manner for the observed effects of substrate concentration, p_H , and salts on the velocity of the catalysed reaction.

It is a pleasure to express my gratitude to Prof. J. B. S. Haldane for his constant and illuminating criticism, and to Sir F. G. Hopkins for his interest and encouragement.

REFERENCES.

- Bertho (1929). *Liebig's Ann.* **474**, 1.
 Dixon and Thurlow (1924). *Biochem. J.* **18**, 976.
 Haldane (1930). *Enzymes*. (Longmans, Green and Co., London.)
 Josephson (1925). *Z. physiol. Chem.* **147**, 1.
 Kuhn (1925). *Oppenheimer's Die Fermente*. (Thieme, Leipzig.)
 Mann and Woolf (1930). *Biochem. J.* **24**, 427.
 Michaelis and Menten (1913). *Biochem. Z.* **49**, 333.
 ——— and Pechstein (1913). *Biochem. Z.* **53**, 320.
 Myrbäck (1926, 1). *Z. physiol. Chem.* **158**, 160.
 ——— (1926, 2). *Z. physiol. Chem.* **159**, 1.
 Quastel (1926). *Biochem. J.* **20**, 166.
 ——— and Whetham (1924). *Biochem. J.* **18**, 519.
 ——— and Wooldridge (1927, 1). *Biochem. J.* **21**, 148.
 ——— (1927, 2). *Biochem. J.* **21**, 1224.
 Sidgwick (1927). *The electronic theory of valency*. (Oxford.)
 Willstätter (1927). *J. Chem. Soc.* 1359.
 Woolf (1929). *Biochem. J.* **23**, 472.

XLIH. ON THE INADEQUACY OF WHITE WHEAT FLOUR AND FISH MEAL FOR PROLONGED GROWTH OF PIGS AND ITS AMELIORATION WITH YEAST OR STOUT.

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INTRODUCTION.

PLIMMER, ROSEDALE and RAYMOND [1927] used a diet of white flour and fish meal with varying quantities of marmite and dried yeast in maintenance experiments on pigeons and chickens. They concluded that the calories in the diet need balancing by vitamin B ($B_1 + B_2$).

The quantity of dried yeast required was found, in the case of chickens, to be 6 to 10 % in a food containing no vitamin B.

Very little work however has been carried out on the requirements of pigs for the yeast vitamins. Gibson and Concepcion [1916] in their experiments on pigs fed exclusively on cow's milk from the age of 17 days, obtained evidence of neuritis by the histological examination of the sciatic and pneumogastric nerves in pigs killed 34 days and 60 days after the start of their experiments, but the large daily intake of a pig has prevented the use of these animals for vitamin B investigations.

The experiments reported below were undertaken to answer the following questions.

(1) If a diet of white wheat flour and fish meal was inadequate for the growth of pigs.

(2) If dried yeast as a recognised and available source of B vitamins would improve the diet.

(3) If a stout which contained some living yeast (see p. 357) had the property of ameliorating a diet deficient in B vitamins.

The pig is able to consume a sufficient quantity of stout and thus the difficulties of previous concentration or extraction, necessary in the case of pigeons and rats, are avoided.

Experiments have, nevertheless, also been carried out with rats, and these will form the subject of another communication.

EXPERIMENTAL.

Five experiments are described below, involving the use of 59 pigs. In all these experiments the animals were divided into comparable groups, all receiving a basal diet of white wheat flour and white fish meal.

The flour was specially ground from English wheat by a local miller and was free from germ and husk.

The chemical composition of the constituents of the basal ration was as follows:

	Wheat flour %	White fish meal %
Moisture	19.85	10.32
Oil	2.98	6.23
Proteins	7.90	63.10
Starch, etc.	68.82	—
Ash	0.45	20.35
	100.00	100.00

A mixture of 90 % flour and 10 % fish meal showed a starch equivalent of 69.05 and an albuminoid ratio 1:5.4; the digestible proteins were 10.94 %. Such a mixture would not from its composition seem unsuitable for pigs and when the pigs are nearing bacon weight the fish meal could be cut down to 5 % of the mixture, which was done in two of the experiments reported below; the albuminoid ratio was then widened to 1:7.30 and the digestible protein reduced to 8.39 %. Although the fish meal might be assumed to supply a sufficiency of the vitamins A and D, cod-liver oil was also given at the commencement of each experiment. The ash in the fish meal was considered ample for mineral requirements.

The pigs in the positive control group received, in addition, dried brewer's yeast at the rate of 2 ounces per pig per day, except in Exp. 4 (see below).

The dried yeast had the following composition:

Moisture	Oil	Proteins	Carbohydrates	Woody fibre	Ash
9.58 %	0.46 %	45.91 %	32.13 %	3.15 %	8.77 %

The stout was fed in varying quantities as given below. It was in good condition and a fresh supply was received in bottles weekly.

The food for each lot of pigs was weighed out every morning and mixed with water in a large covered earthenware jar.

With the exception of Exps. 3 and 4 the dried yeast was mixed with the food in the morning. In these experiments the yeast was mixed at the time of feeding.

The stout was always mixed with the food at feeding time. The pigs were fed at 8 a.m. and 4 p.m. each day. They were weighed every Tuesday at noon.

A short detailed account of the experiments is given below. Although these differ in minor details the first three may be regarded as repeat experiments, and the results in general are confirmed by Exps. 4 and 5 as will be seen by reference to the charts, Figs. 1 and 2.

In Exp. 4 the negative control group repeated previous experiments but

the other two groups only differed from them in that half an ounce daily of dried brewer's yeast was given per pig in one lot and the equivalent of $\frac{1}{4}$ ounce per pig as a water extract daily in the other group.

In Exp. 5 the negative control group repeated previous experiments, but the three other lots differed from them in that the mixture of flour and fish meal was cooked by blowing steam into the pails in which it had been weighed out.

Exp. 1. After a preliminary period of 1 week to accustom the animals to the diet the experiment was started on January 22nd, 1929. A selection was made from two litters, 8 Large White pigs 73 days old and 4 Large White \times Middle White pigs 87 days old being chosen.

10 cc. of cod-liver oil were given to each pig daily for the first 55 days of the experiment. The ration was altered to 95 % flour and 5 % fish meal on the 74th day.

The bedding of the animals was changed on April 9th from cereal straw to wood shavings, as it was thought that they were eating enough straw to make a possible difference to the results. This change apparently did not make any difference.

The group shown at the bottom of Fig. 1 received half a pint of stout per pig per day until the 80th day when the quantity was increased to 1 pint.

Exp. 2. This experiment was started on April 30th, 1929. Ten Large White \times Middle White pigs all from one litter were available. They were 79 days old at the start of the experiment. The pigs were divided into groups of 4, 3 and 3.

Cod-liver oil was given to all the pigs until the 39th day at the rate of 10 cc. per pig per day. On the 51st day the basal diet was altered to 95 % wheat flour and 5 % fish meal. Wood shavings were used as bedding throughout the experiment.

The pigs in the third group received 1 pint of stout per day.

Exp. 3. This experiment was started on February 18th, 1930. Purchased pigs of unknown origin were used. The pigs were divided into 4 groups of 3 pigs each. Two groups were kept as controls, one received stout and one received 2 ounces of dried brewer's yeast.

Cod-liver oil was given to all the pigs from February 26th to March 26th at the rate of 10 cc. per pig per day, after which date only the pigs in one of the negative control groups (group No. 1) continued to receive the oil.

The pigs did not have any bedding in this experiment.

Exp. 4. This experiment was started on June 25th, 1930. Eleven Middle White \times Large White pigs were available; they were farrowed on the Institute's farm on April 14th, 1930.

They were divided into three pens as follows:

No. 1. 1 sow and 2 hogs fed on 90 % flour 10 % fish meal.

No. 2. 1 sow and 3 hogs fed on 90 % flour 10 % fish meal + $\frac{1}{2}$ oz. yeast.

No. 3. 1 sow and 3 hogs fed on 90 % flour 10 % fish meal + yeast extract.

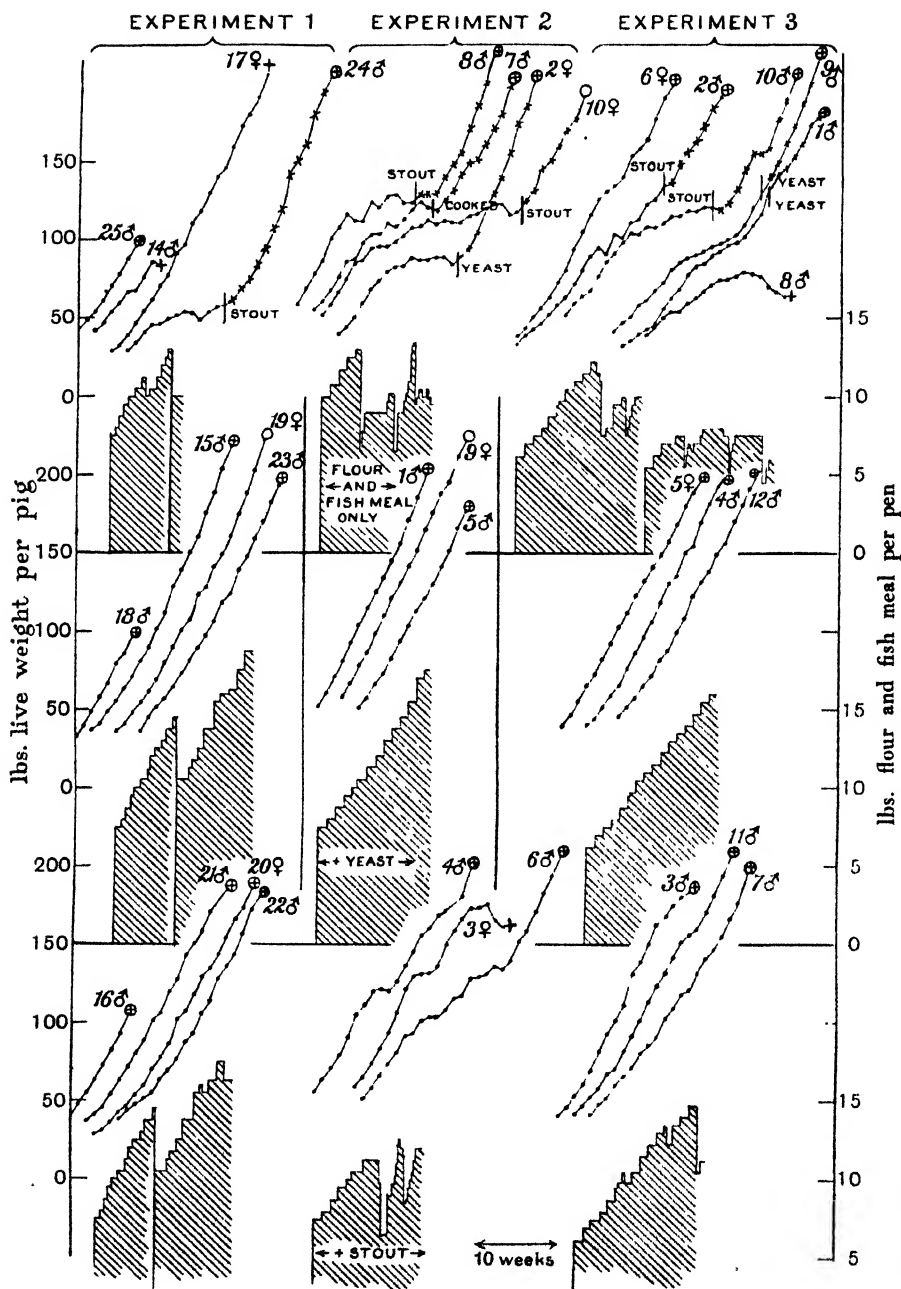


Fig. 1.

Figs. 1 and 2. Results of Exps. 1 to 5 are shown in vertical columns. Growth curves of individual pigs in lb. live weight are arranged above the daily intake of flour and fish meal per pen. The growth curves and intake records of each experiment started on the same day but have been spaced to avoid overlapping. The terminal signs at the end of each growth curve indicate
 ⊕ = Pigs sent to butcher. + = Pig died. ○ = Pig removed from experiment.

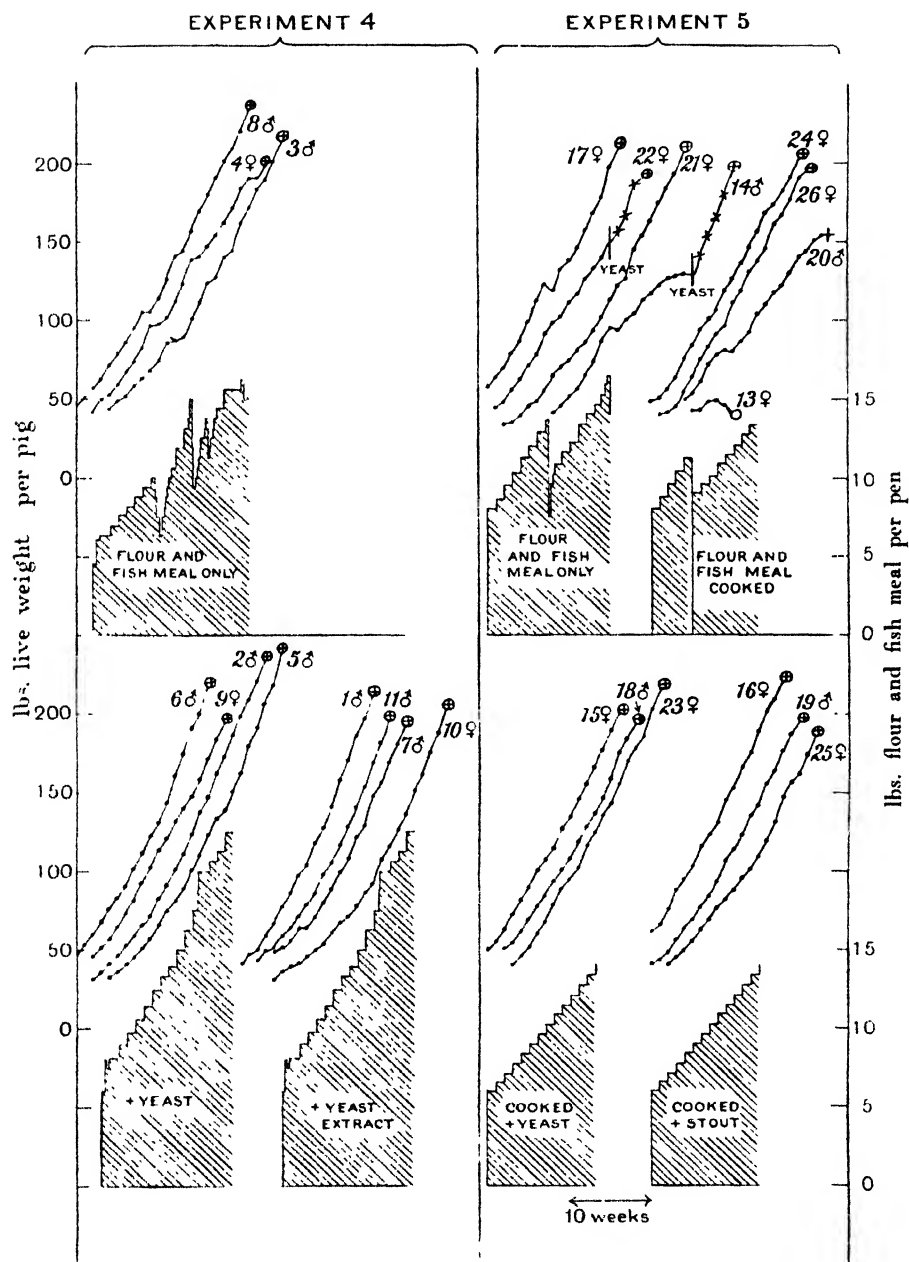


Fig. 2.

Curative treatment of pigs in negative control groups started at the vertical line, the nature of the treatment being indicated.

The top sets of growth curves and intake diagrams in both figures are negative controls, flour and fish meal only.

In the lower sets yeast and stout were added as shown.

The yeast extract was made by pouring boiling water on dried brewer's yeast; after stirring for 1 minute the infusion was left to cool and decanted 1 hour later through a cheese cloth. The liquid was squeezed out and after standing for a day was again decanted from sediment.

The total solids in the extract averaged 2.75 %; 74 cc. of this extract derived from 7.4 g. (= 0.26 oz. and containing 2.03 g. dry extracted matter) were fed to each pig daily. The yeast extract required to maintain the growth of a rat on a diet free from vitamin B was 0.5 cc. per rat daily. No bedding was given to the pigs in this experiment.

The yeast extract was not taken readily at first and had to be increased little by little to an amount equivalent to one quarter of an ounce of the original yeast per pig per day.

Exp. 5. This experiment was started on March 4th, 1930. Fourteen purchased pigs of unknown origin and mixed breed were used; they were similar to those used in Exp. 3. They were divided into 4 lots as follows:

No. 1. 3 sows and 1 hog fed on 90 % flour and 10 % fish meal uncooked.

No. 2. 3 sows and 1 hog fed on 90 % flour and 10 % fish meal cooked.

No. 3. 2 sows and 1 hog fed on 90 % flour and 10 % fish meal cooked + 2 oz. yeast.

No. 4. 2 sows and 1 hog fed on 90 % flour and 10 % fish meal cooked + stout.

As in Exp. 3 the pigs had no other bedding than boards in their sleeping pens.

RESULTS.

The growth curves of the pigs are set out in Figs. 1 and 2 with the intake of each group. The comparable groups in each experiment are arranged in vertical columns.

When Exp. 1 had been in progress 48 days there was little to choose between the groups and it was thought that negative results only might be obtained. One pig from each group was, therefore, sent to the butcher as a "porker." The food intake of the pen was reduced at this date in proportion to the weight of the pig removed, this is indicated by a break in the intake graph.

In all the experiments the differences between the weights of the individual pigs in the three groups were not very marked at the end of the first 40 to 50 days on the diet. The exceptions were pig No. 24 in Exp. 1 which did not thrive on the flour and fish meal diet and pig No. 13 in Exp. 5 which did not grow on the cooked flour and fish meal and had to be taken out of the experiment as it was feared it was developing an illness.

If we had sent the pigs to the butcher at porker weight the results would have been indefinite. By keeping the pigs on to bacon weight of 200 lb. it becomes quite clear that there are marked differences in the development of the three groups.

In every group receiving 2 oz. of yeast the growth was very uniform and the pigs seemed in good health. The pigs receiving stout exhibited greater differences between individual members of the same group, but were also healthy and free from sickness (except sow No. 3, Exp. 2, which died from generalised peritonitis). They were, however, much more uniform in growth and better in health and appearance than the negative controls.

The negative control groups showed features which have not infrequently been seen in experiments of this character, namely that whereas some of the pigs grew satisfactorily others suffered from lack of appetite, sickness, and in some cases death.

Reference to the charts shows that the growth was irregular, some pigs doing better than others. The growth alone does not give an adequate picture of the unthrifty rough-coated appearance of these negative control pigs.

It is necessary to discuss these animals in greater detail and to give some particulars of the *post mortem* findings.

There were 11 sows and 14 hogs fed on a diet of flour and fish meal only. Of these 3 hogs and 1 sow died, 8 hogs and 3 sows were cured (see below) 1 sow was removed from the experiment and 3 hogs and 6 sows survived without treatment.

All the pigs which were sent to the butcher (marked \oplus on charts) and all pigs which died (marked + on charts) were submitted to *post mortem* examinations and in some cases bacteriological and histological examinations were also made.

In this work we are greatly indebted to Mr W. L. Little for *post mortem* examinations, and Dr J. Mills (Pathologist to the Berkshire Hospital) for histological examinations.

In this preliminary note it is not proposed to go into details but Dr Mills reports that "there was no morbid anatomical appearance sufficiently typical of vitamin B deficiency disease to enable one to state definitely from the *post mortem* examination that death was due to such deficiency.

In some of the *post mortem* examinations the sciatic nerves on histological examination showed extravasation of blood into the connective tissue between the bundles of nerve fibres but it is doubtful whether this was due to disease or an artefact produced in removing the nerves, in any case the nerve fibres themselves appeared normal."

Curative treatment.

After varying periods on the basal diet, stout or dried yeast was given to a number of the negative control pigs which failed to grow. This caused a marked increase in the rate of growth in all cases, as can be seen in Table I.

In Exp. 2, hog No. 7 was given cooked food when, after 102 days on uncooked food, its weight began to decline as is shown in Table I and Fig. 1. The rate of growth of this pig improved from the time of the change of diet. In Exp. 5, however, where cooked food was given from the commencement

Table I. *Curative treatment.*

Exp. No.	Pig No.	Sex	Wt when curative treatment of negative controls commenced	6 weeks before treatment		6 weeks following treatment	
				Diet Flour and fish meal daily	lbs. live wt gain per day	Diet Flour and fish meal daily	lbs. live wt gain per day
1	24	Hog	58.5	Only	0.167	+ 1½ pints stout	1.155
	23	"	57	+ 2 oz. yeast	0.952*	+ 2 oz. yeast	1.155
2	2	Sow	84	Only	-0.012	+ 2 oz. yeast	1.333
	7	Hog	121.5	"	0.417	Cooked	0.690
	8	"	124	"	0.286	+ 1½ pints stout	0.774
	10	Sow	118.5	"	0.060	+ 1½ pints stout	1.202
	1	Hog	128	+ 2 oz. yeast	1.548	+ 2 oz. yeast	1.762
	9	Sow	121.5	+ 2 oz. yeast	1.500	+ 2 oz. yeast	1.583
3	1	Hog	132	Only	0.917	+ 2 oz. yeast	1.095
	2	"	135	"	0.738	+ 2½ pints stout	1.190
	9	"	130	"	0.798	+ 2 oz. yeast	1.667
	10	"	122	"	0.274	+ 2½ pints stout	0.786
	4	"	130	+ 2 oz. yeast	1.524	+ 2 oz. yeast	1.500
5	14	Hog	129	Only	0.381	+ 2 oz. yeast	1.857*
	22	Sow	151	"	1.167	+ 2 oz. yeast	1.429*
	18	Hog	128	Cooked + 2 oz. yeast	1.190	Cooked + 2 oz. yeast	1.580
	15	Sow	146	Cooked + 2 oz. yeast	1.333	Cooked + 2 oz. yeast	1.548*

* Calculated on a shorter period than 6 weeks.

The figures in heavy type are given for comparison. They represent pigs from the same litter in each experiment fed with an addendum of 2 oz. of yeast from the start. They are taken from the weighing at which they most nearly corresponded to pigs in the negative control groups.

of the experiment there appeared to be no marked improvement in favour of the cooked food when compared with a control group of pigs receiving uncooked food.

DISCUSSION.

All five experiments indicated that the basal diet was inadequate for the nutrition of pigs for a prolonged period.

At the start and for periods of about 7 weeks the pigs on the basal diet grew well and consumed almost as much food as the pigs in the other lots. In the earlier part of the experiment 2 oz. extra food were offered to balance the yeast given to other pigs.

It was frequently recorded that, even when the negative control pigs received less food than those in the other two pens, they did not clear up their allowance. When this happened the quantity was reduced, for we have always found that if pigs are offered more than they require their appetite goes off. They were, however, always tempted with more food as soon as they had cleared up the quantity given. In Exp. 4 the food refused was dried and weighed. The regularity and uniformity of the growth and food consumption in the lots receiving yeast are most striking when compared with the negative controls. The pigs receiving stout come between the two groups. The pigs on stout received less dry matter in the supplement than the yeast-fed pigs.

The quantity of yeast they could have received in the stout was about 1/1000th that supplied by 2 ozs. of dried yeast.

SUMMARY.

A basal diet of wheat flour and fish meal has proved inadequate for the sustained growth of pigs.

Additions of 2 oz. per pig daily of dried brewer's yeast made the diet quite satisfactory. In Exp. 4, $\frac{1}{2}$ oz., or even extract equal to $\frac{1}{4}$ oz., of yeast made the diet satisfactory. Stout given in quantities varying from $\frac{1}{2}$ pint to 2 pints per pig daily had a similar but less marked effect.

We wish to express our indebtedness to Lord Iveagh for a grant which has made it possible to undertake work on this and other problems of nutrition, and to Messrs Arthur Guinness, Son and Co. for supplying the stout and dried yeast.

REFERENCES.

- Gibson and Concepcion (1916). *Philipp. J. Sci.* B 11, 126.
Plummer, Rosedale and Raymond (1927). *Biochem. J.* 21, 930.

XLIV. THE FAT-SOLUBLE VITAMIN REQUIREMENTS OF THE CHICK.

I. THE VITAMIN A AND VITAMIN D CONTENT OF FISH MEAL AND MEAT MEAL.

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IN a recent communication [McFarlane, Graham and Hall, 1930] it was shown that normal chicks could not be grown in confinement when fed synthetic diets which supplied what are believed to be adequate amounts of the vitamins known to be essential for growth in the chick. The birds during the 4th to the 6th week of growth developed certain deformities and paralysis of the limbs, a condition commonly designated as leg-weakness, the cause of which could not be adequately explained by what is now known of the nutritional requirements of the chick. Much difference of opinion exists in the literature in regard to the cause of this pathological condition, which has long been the greatest difficulty in rearing chicks in the laboratory on synthetic diets. It would appear either that more than one dietary factor may be responsible for this condition, or that there is more than one form of leg-weakness. One type of leg-weakness studied by many workers, notably Hughes and Titus [1926]; Mitchell, Kennard and Card [1923]; Hart, Halpin and Steenbock [1922]; Emmett and Peacock [1923] and more recently by Mussehl and Ackerson [1930] and by Massengale and Nussmeier [1930], can be prevented by the addition of cod-liver oil to the diet. As to whether this effect is due to vitamin A or vitamin D is still disputed. The preponderance of evidence indicates that it is a vitamin A deficiency which is the cause of this particular type of leg-weakness and that this leg deformity is not a rachitic condition [see Pappenheimer and Dunn, 1925]. All these investigators agree that the fat-soluble vitamin requirements of the chick are high and that to prevent the incidence of this form of leg-weakness, in chicks fed in the laboratory, at least 2 % of a high grade cod-liver oil must be added to the diet.

Plimmer, Rosedale and Raymond [1927] believe they have confirmed the findings of Dunn [1924] that as little as 0.5 % of cod-liver oil of good quality is sufficient to rear chicks to maturity. They obtained little or no growth and lost all the chicks within 4 weeks on feeding a diet composed of white rice

78 %, dried yeast 17 %, and fish meal 4 %. The addition of 0.4 or 0.6 parts of cod-liver oil (their Group XXXII A), did not materially improve the results. On changing the diet to white rice 75.3 %, dried yeast 16 %, fish meal 8 %, and cod-liver oil 0.5 % (their Group XLIII), excellent growth was obtained. They themselves express surprise that "birds could be reared on such small quantities of cod-liver oil as 0.1 and 0.2 %." Again with a diet composed of maize 89 %, fish meal 10 %, and cod-liver oil 1 %, normal rearing was obtained, but when the amount of cod-liver oil was reduced to 0.25 % or no cod-liver oil was given, rickets developed. The chicks, however, appear to have grown fairly well and only one chick was lost when the amount of cod-liver oil was as little as 0.25 %. From these results they have concluded that as little as 0.5 % of cod-liver oil of good quality is sufficient to rear chicks to maturity. Their conclusion is evidently based on the assumption that the fish meal contained no fat-soluble vitamins or possibly the fish meal had been freed from fat by extraction with ether, but this is not mentioned in their paper. The only essential difference between the diet fed to Group XXXII A which was a failure and that fed to Group XLIII which grew exceptionally well was in the fact that twice the quantity of fish meal was fed to the latter group; this improvement, we presume, they attributed entirely to the increase in the percentage protein in the diets. Again with the diet containing large quantities of maize, the failure of 10 % fish meal to protect the birds against rickets when no cod-liver oil was added may not indicate that fish meal contains little or no vitamin D but rather that maize compared with white rice, as shown by Mellanby [1929], contains a large amount of the anticalcifying factor which interferes with the deposition of calcium salts.

That fish meal may contain a very considerable amount of the fat-soluble vitamins while meat meal is not so endowed was suggested to us by the experiments of Graham and Smith [1929]. In a study of the influence of various protein materials on reproduction in the chick these workers found that the addition of 2 % of cod-liver oil to a diet for laying hens, containing 10 % of meat meal, had a pronounced effect in increasing the hatchability of the fertile eggs. The same basal diet but with 7.5 % of white fish meal as the protein supplement and without cod-liver oil gave just as high a percentage hatchability of the fertile eggs as that obtained with the meat meal diet containing 2 % cod-liver oil. The addition of 2 % cod-liver oil to the fish meal diet resulted in little or no improvement in the hatchability of the eggs.

The experiments to be described were undertaken to determine whether the vitamin A and vitamin D content of the fat of fish meal was different from that of the fat of meat meal. The results show conclusively that there is a difference, *i.e.* the particular white fish meal used in these experiments contained a very considerable amount of both vitamin A and vitamin D. In view of this the results of Plimmer *et al.* [1927] might legitimately be interpreted as showing that the vitamin A or vitamin D requirements of the chick are high and that the amount of vitamin A and vitamin D in the quantity of

fish meal fed in their experiments, while quite considerable, was not sufficient to protect the birds against rickets or a vitamin A deficiency, but that complete protection and normal growth were secured by adding 0.5 % cod-liver oil. Their findings, which constitute the most recent attempt recorded in the literature to assay the fat-soluble vitamin requirements of the growing chick, cannot, in the light of the results reported below, be considered reliable unless they have taken the precaution to remove by ether extraction the vitamin A and vitamin D which the fish meal used in their basal diet undoubtedly contains. Since this is not recorded in their paper, it can only be assumed that the fish meal added to their diet a considerable amount of the fat-soluble vitamins and that the vitamin A and vitamin D requirements of the chick may be relatively high instead of, as they have concluded, remarkably low.

EXPERIMENTAL.

The fish meal used in these experiments was a white fish meal prepared from non-oily fish such as cod (from which the liver had been removed) hake, plaice, haddock, skate and ling, etc. The meat meal was a finely ground meaty substance consisting of the residues of animal tissues but exclusive of hoof and horn and from which the fat had been partially extracted by hydraulic pressure after the direct drying of the material in revolving drums under 40 lbs. steam pressure (240°). The content of ether-soluble substances in fish meal is relatively low, while that in meat meal is much greater (see Table I). The cod-liver oil used was a high grade medicinal oil obtained from W. A. Munn, St John's, Newfoundland.

Table I. *Percentage composition of fish meal and meat meal.*

	Fish meal	Meat meal
Moisture	5.90	3.84
Crude protein (N \times 6.25)	75.77	55.90
Ether extract	3.86	14.58
Crude fibre	0.23	1.22
Total ash	17.63	19.51
Silica	1.90	0.44
P ₂ O ₅	5.55	6.85
CaO	5.97	8.55
MgO	0.88	0.59
Na ₂ O	1.81	1.02
K ₂ O	0.48	0.44
Fe ₂ O ₃	0.016	0.04
Cu (mg. per kg.)	4.20	5.40
Ratio calcium : phosphorus	1.6 : 1	1.9 : 1

To ascertain whether fish meal or meat meal contains considerable amounts of vitamin A or vitamin D, the rates of growth of nine groups of barred rock chicks from 1 day to 8 weeks of age on the following diets were determined.

Fish meal groups.

Group I. Fish meal 14.8 %, marmite 15 %, white rice 70.2 %.

Group II. Same diet but with 2 % cod-liver oil replacing an equal amount of white rice.

Group III. Same diet as Group I, but with the chicks irradiated for 15 minutes daily from a quartz mercury vapour lamp at a height of 24 inches.

Group IV. Fish meal (freed from fat by extraction with ether) 14.6 %, marmite 15.0 %, white rice 67.4 %, and cod-liver oil 3 %.

Meat meal groups.

Group V. Meat meal 16.6 %, marmite 15 %, white rice 68.4 %.

Group VI. Same diet as group V but with 2 % cod-liver oil replacing an equal amount of white rice.

Group VII. Same diet as group V but with the chicks receiving ultra-violet irradiation for 15 minutes daily.

Group VIII. Meat meal (freed from fat by extraction with ether) 14.8 %, marmite 15.0 %, white rice 67.4 %, and cod-liver oil 3 %.

Control group.

Group IX. Caseinogen 12.2 %, marmite 15 %, white rice 65.8 %, cod-liver oil 3 %, and salt mixture 4 % [see Hart *et al.*, 1920].

The total protein (N \times 6.25) of all these diets was approximately 18.5 %, being made up of 5.8 % from marmite, 2.6 % from white rice, and 10 % from each of the protein sources. The total ash of all the diets was approximately 4.4 %. The experiment was commenced with day-old chicks, 35 in each group, housed and managed in the laboratory under the same conditions as described by McFarlane, Graham and Hall [1930]. At the end of the 1st week the numbers were reduced to 30 chicks in each group, discarding those either showing evidence of constitutional weakness, or varying most widely from the mean weight of the group.

In Fig. 1 and Fig. 2 is shown the average growth in g. of the chicks receiving the different fish meal and meat meal diets. The weekly mortality in each group is also recorded on each growth curve in arabic figures, each value representing the number of chicks which died during the previous week. The growth of the control Group IX receiving caseinogen as the source of protein is recorded in each figure for comparison. No chicks were lost in this group during the first 8 weeks of growth.

We have also determined the calcium and inorganic phosphorus content of the blood-serum of a number of chicks in each group with the exception of Groups VII and VIII. In Fig. 3 and Fig. 4 are plotted the results of these determinations. The analyses at the end of the 1st week were made on the chicks which were being discarded from the experiment, it being found necessary to combine the blood from two chicks on the same diet to obtain sufficient blood for a determination. It was necessary to sacrifice two or three chicks in each group each week until the 8th, when sufficient blood for the analysis could be obtained from the brachial artery without the loss of the chick. Calcium was determined on 2 cc. of serum by the Clark-Collip modification [1925] of the Kramer-Tisdall method [1921]; inorganic phosphorus was determined on the supernatant fluid from the serum-calcium by the

Gunther-Greenberg modification [1929] of the Fiske and Subarrow procedure [1925]. For comparison we have also recorded the results of calcium and inorganic phosphorus determinations on the serum of chicks in the control caseinogen Group IX, of chicks receiving a rachitic diet and housed behind window-glass (Group X), and of chicks receiving the same diet as Group X but with 2 % of cod-liver oil added and housed under vita-glass (Group XI). The diet on which the chicks developed rickets was composed of yellow corn 37 %, ground wheat 50 %, oatmeal 25 %, buttermilk powder 10 %, meat

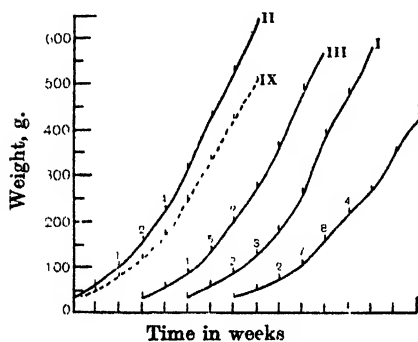


Fig. 1.

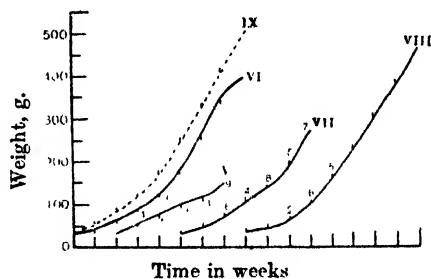


Fig. 2.

Fig. 1. The effect on growth of various additions to a diet composed of fish meal 14.8 %, marmite 15 %, and white rice to 100.

- Curve I. No cod-liver oil added or ultra-violet irradiation.
 " II. 1 % cod-liver oil added.
 " III. Chicks received ultra-violet irradiation 15 min. daily.
 " IV. Ether-extracted fish meal and 3 % cod-liver oil.
 " IX. Caseinogen control diet and 3 % cod-liver oil.

NOTE. The arabic figures on each group curve in Figs. 1 and 2 give the number of chicks which died during the preceding week.

Fig. 2. The effect on growth of various additions to a diet composed of meat meal 16.6 %, marmite 15 %, and white rice to 100.

- Curve V. No cod-liver oil added or ultra-violet irradiation.
 " VI. 1 % cod-liver oil added.
 " VII. Chicks received ultra-violet irradiation 15 min. daily.
 " VIII. Ether-extracted meat meal and 3 % cod-liver oil.
 " IX. Control caseinogen diet and 3 % cod-liver oil.

meal 12.5 %, alfalfa meal 3.5 %, bone meal 2.5 %, oyster shell 1.5 %, and salt 0.5 %.

DISCUSSION.

During the first eight weeks of growth the inorganic phosphorus content of the blood-serum of normal chicks (Groups IX and XI, Fig. 4) is fairly constant but with chicks receiving a rachitic diet (Group X) the inorganic phosphorus content of the serum drops to a very low level during the 3rd and 4th weeks, when the birds show a typical rachitic condition, and then slowly recovers to the original level. These changes in the inorganic phosphorus of the blood-serum of rachitic chickens are similar to those accompanying uncomplicated mammalian rickets.

It will be seen from Fig. 1 that good growth and very little mortality was obtained with a diet containing fish meal as the only source of the fat-soluble vitamins (Group I) and that the result was practically unchanged when chicks on the same diet received ultra-violet irradiation (Group II). The growth was,

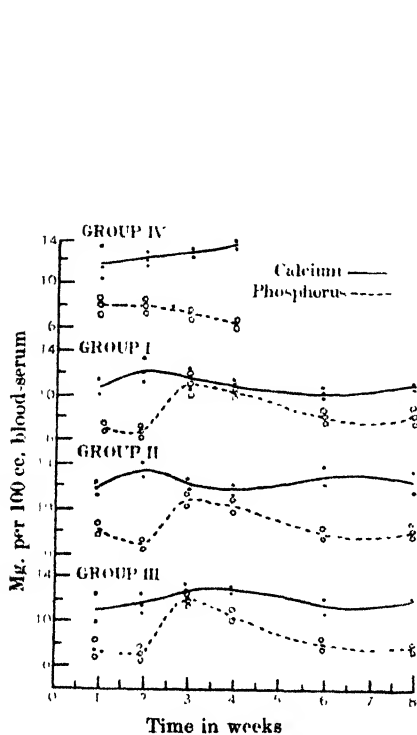


Fig. 3.

Fig. 3. Showing the effect on the calcium and inorganic phosphorus content of the blood-serum of chicks fed different fish meal diets.

- Group I. No cod-liver oil. Group II. 1 % cod-liver oil.
 " III. Ultra-violet irradiation 15 min. daily.
 " IV. Fish meal ether-extracted and 3 % cod-liver oil.

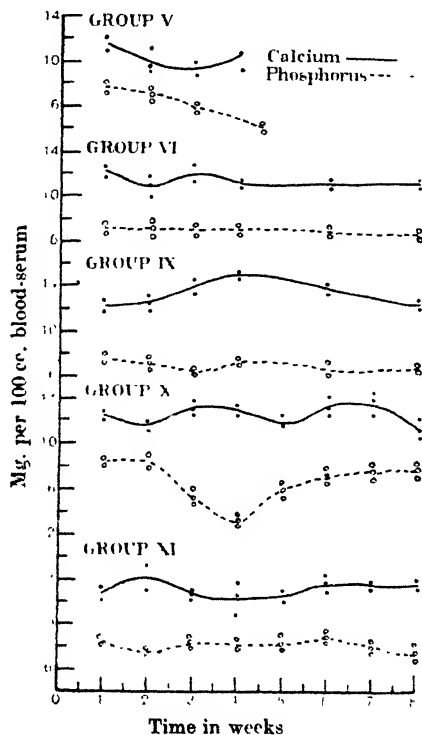


Fig. 4.

Fig. 4. The effect on the calcium and inorganic phosphorus content of the blood-serum of chicks fed different meat meal diets.

- Group V. No cod-liver oil. Group VI. 1 % cod-liver oil.
 " IX. Caseinogen control diet, 3 % cod-liver oil.
 " X. Rachitic diet, no cod-liver oil.
 " XI. Same diet as Group X but with 2 % cod-liver oil.

however, improved by adding 1 % cod-liver oil to the diet and was better than that attained by the caseinogen control Group IX. Fish meal which had been extracted with ether (Group IV) gave much the poorest growth of all notwithstanding the fact that 3 % cod-liver oil was added. It will also be observed that the mortality was very high with Group IV particularly during the 3rd and 4th weeks of growth. 50 % of these losses were the result of haemorrhage following the insertion of the identification bands into the wing.

Bleeding from the small wound slowly continued for 12 to 24 hours, the feather areas were continually wet with blood, the birds ultimately bleeding to death. The remainder of the losses all showed large sub-dermal haemorrhages along the femur (particularly the left), ribs and pectoral muscles and low blood volume due to haemorrhage. Blood from chicks in this group failed to clot on standing overnight in the laboratory. This condition was characteristic of the feeding of fish meal and to a less extent of meat meal which had been extracted with ether, no losses due to haemorrhage being recorded when caseinogen or untreated fish meal or meat meal was fed, although these chicks were all wing-banded at the same time and in exactly the same manner.

Coincident with the fact that the blood of the chicks fed ether-extracted fish meal would not clot there was also a distinct drop in the inorganic phosphorus content of the blood-serum (Fig. 3, Group IV) at the 4th week of growth. Unfortunately the losses in this group were so great that the blood-serum calcium and phosphorus determinations had to be discontinued at the end of the 4th week. However, the drop in the inorganic phosphorus is significant, particularly when it is noted (Fig. 3) that there was a very pronounced increase in the inorganic phosphorus content of the blood-serum during the 3rd and 4th weeks of growth of the chicks in all the groups fed unextracted fish meal. Since some factor other than the vitamin D, calcium or inorganic phosphorus content of the diet of the chick may have a profound effect on the concentration of inorganic phosphorus in the blood-serum during the first 8 weeks of growth, the value of this determination as a criterion of the presence or absence of rickets or leg-weakness in chicks is extremely questionable. It would seem that the removal of phospholipins from the fish meal was responsible for the result with Group IV but this does not explain the failure of the blood to coagulate, as the caseinogen of the control Group IX was also ether-extracted and the clotting time of the blood of the chicks was quite normal.

The results with these groups show that a diet containing approximately 15 % of this particular white fish meal as the only source of the fat-soluble vitamins contained sufficient vitamin A and vitamin D to give normal growth with no external evidence of either leg-weakness or rickets. The addition of 1 % cod-liver oil to this diet increased the rate of growth, presumably by increasing the vitamin A content of the diet, as increasing the concentration of vitamin D by ultra-violet irradiation had no significant effect.

With the meat meal groups (Fig. 2) the growth when no cod-liver oil was added (Group V) was extremely poor and all the chicks died within 5 weeks. The inorganic phosphorus content of the blood-serum of the chicks in this group fell rapidly during the first 4 weeks (Fig. 4, Group V). The chicks, however, did not grow sufficiently to show any external evidence of rickets. Giving the chicks ultra-violet irradiation resulted in an improved growth which, however, was still distinctly sub-normal and all the chicks were lost

within 6 weeks (see Fig. 2, Group VII). Adding 1 % cod-liver oil to the meat meal diet (Group VI), while greatly improving the results, still gave sub-normal growth when compared with the caseinogen control Group IX. Since normal growth was obtained with Group VIII receiving ether-extracted meat meal and 3 % cod-liver oil it does not seem probable that the nature of the protein or inorganic constituents was responsible for the sub-normal growth of Group VI. There is no significant difference in the composition of the ash of fish meal and meat meal so far as our analyses have been completed (see Table I). There were no external evidences of rickets in the chicks in Groups VI, VII and VIII. The inorganic phosphorus content of the serum of the chicks in the meat meal Group VI receiving 1 % cod-liver oil was constant throughout the first 8 weeks of growth.

The results with these groups show that a diet containing approximately 16.5 % of this particular meat meal as the sole source of the fat-soluble vitamins did not contain sufficient vitamin A or vitamin D to sustain growth. Since the addition of 1 % cod-liver oil to this diet still resulted in sub-normal growth and since normal growth was obtained when 3 % cod-liver oil was added to a diet containing ether-extracted fish meal it would appear that 1 % of high-grade medicinal cod-liver oil did not supply sufficient vitamin A or vitamin D to promote normal growth. Assuming the meat meal to be free from fat-soluble vitamins, the amount of vitamin A or vitamin D required to promote normal growth in chicks up to 8 weeks of age is, according to these results, in excess of the amount supplied by 1 % of high grade medicinal cod-liver oil.

Further work is being commenced to establish more accurately the vitamin A and vitamin D requirements of the chicks by varying the amount of cod-liver oil in the simplified caseinogen diet which has been used as a control in these experiments.

SUMMARY.

1. A sample of white fish meal when fed at a level of 15 % in a diet composed of marmite and white rice was found to contain sufficient vitamin A and vitamin D to promote normal growth of chicks until 8 weeks of age. A sample of meat meal was found to contain little or no vitamin A or vitamin D when compared with fish meal.

2. The conclusion of Plimmer *et al.* [1927], that as little as 0.5 % of cod-liver oil of good quality is sufficient to rear chicks to maturity is based on the assumption that the fish meal used in their diets contained little or no fat-soluble vitamins. This assumption, for reasons discussed in the text, is believed to be erroneous. An experiment is described which shows that the amount of high grade medicinal cod-liver oil required to rear chicks, in the laboratory, to 8 weeks of age is in excess of 1 %.

3. Some factor, other than the vitamin D, calcium or inorganic phosphorus content of the diet of the chick, has been found profoundly to affect

the concentration of inorganic phosphorus in the blood-serum of chicks during the first 8 weeks of growth.

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REFERENCES.

- Clark and Collip (1925). *J. Biol. Chem.* **63**, 1925.
 Dunn (1924). *J. Biol. Chem.* **61**, 129.
 Emmett and Peacock (1923). *J. Biol. Chem.* **56**, 679.
 Fiske and Subarrow (1925). *J. Biol. Chem.* **66**, 375.
 Graham and Smith (1929). *Report to the British Empire Marketing Board, London.*
 Gunther and Greenberg (1929). *J. Biol. Chem.* **82**, 551.
 Hart, Halpin and Steenbook (1920). *J. Biol. Chem.* **43**, 423.
 ——— (1922). *J. Biol. Chem.* **52**, 379.
 Hughes and Titus (1926). *J. Biol. Chem.* **69**, 289.
 Kramer and Tisdall (1921). *J. Biol. Chem.* **47**, 475.
 McFarlane, Graham and Hall (1930). *J. Nutrition.* (In press.)
 Massengale and Nussmeier (1930). *J. Biol. Chem.* **87**, 423.
 Mellanby (1929). *Med. Res. Council. Special Report No. 140*, p 147.
 Mitchell, Kennard and Card (1923). *Poultry Sci.* **2**, 117.
 Mussehl and Ackerson (1930). *Poultry Sci.* **9**, 334.
 Pappenheimer and Dunn (1925). *J. Biol. Chem.* **66**, 717.
 Plimmer, Rosedale and Raymond (1927). *Biochem. J.* **21**, 940.

XLV. THE MODE OF ACTION OF VITAMIN D. STUDIES ON HYPERVITAMINOSIS D. THE INFLUENCE OF THE CALCIUM-PHOSPHATE INTAKE.

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THE production of toxic symptoms by overdoses of irradiated ergosterol was first recorded by Pfannenstiel [1927] and Kreitmair and Moll [1928]. A number of workers were unable to confirm this result [Dixon and Hoyle, 1928; Hoyle, 1929; Hoyle and Buckland, 1929; Comel, 1929; Cartland, Speer and Heyl, 1929; Lesné and Clément, 1929] and discredit was thrown by some on the theory of hypervitaminosis, it being suggested that the ill-effects in question must have been caused by toxic by-products, formed it was supposed from the alcohol used as solvent during the process of irradiation [Dixon and Hoyle, 1928; *cf.* Underhill, 1928]. Evidence in support of the opposing view that excess of vitamin D is toxic *per se*, so that the phenomenon genuinely merited the description of hypervitaminosis, was brought forward by Harris and Moore [1928; 1929. 1]. Without denying the possibility that secondary products might also be toxic, it was shown that the severity of the ill-effects ran parallel with the amount of vitamin ingested, that specimens of ergosterol irradiated in various solvents or no solvent were equally toxic when fed at the same level of antirachitic activity, that destruction of vitamin D by over-irradiation entailed a corresponding loss of toxicity, and that transformation products of ergosterol produced by resinification without irradiation were devoid of both antirachitic activity and of toxicity. These conclusions have since been confirmed by the experiments of other workers [*e.g.* Scheunert and Schieblich, 1929]².

¹ In the whole time service of the Medical Research Council.

² Holtz and Schreiber [1930] similarly find that all preparations of irradiated ergosterol which display any antirachitic activity always possess a parallel degree of toxicity, and they are unable to obtain antirachitic but non-toxic products. On the other hand, they claim that they have been able to destroy the antirachitic activity and leave unimpaired the toxic (or "calcinosing") factor. However, the toxicity of the supposed non-antirachitic preparations was typified by its power of raising the blood-P or -Ca (*e.g.* in tetany) and (by definition) of producing calcification. Since these very effects are not infrequently taken as actual indices of antirachitic action, it is difficult to see how the positive result for toxicity, which was demonstrated by tests on dogs, can be reconciled with the reported negative result for antirachitic activity, obtained by tests on rats. Possibly the technique adopted (prophylactic method) may explain the anomaly. It may be

Perhaps the most convincing evidence as to the reality of hypervitaminosis accrued from a consideration of the very nature of the abnormalities produced. We argued [Harris and Moore, 1928; 1929, 1] that since in hypovitaminosis D (*e.g.* in rickets) the typical defective calcification at the growing end of the bone is associated with a diminished blood-P or -Ca, or both, so we might reasonably anticipate finding the reverse condition in hypervitaminosis D, where the characteristic lesion is an excessive calcification of the bone ending [Harris and Innes, 1928-1929], arteries, non-striated muscle¹, *etc.* [Kreitmain and Moll, 1928; Harris and Moore, 1929, 1]. The prediction was found to be justified, the administration of excess of vitamin D giving rise to hyperphosphataemia or hypercalcaemia or both in experimental animals [Harris and Stewart, 1929]. (This result has been confirmed in an extended series of tests [see Harris, 1930] and also by other workers. Hypercalcaemia or hyperphosphataemia has been seen likewise in infants receiving excessive doses of vitamin D [Hess, Lewis and Rivkin, 1928], while Hess, Weinstock and Rivkin [1928] had indeed earlier recorded that the blood-Ca reduced to a low level by a low-Ca, high-P diet could be brought back to a more normal figure by administration of very large amounts of irradiated ergosterol.) In the second place we suggested that just as the low blood-P or -Ca of rickets appeared to be associated with a diminished net absorption from the gut [see Harris, 1928] so again with very large doses of vitamin D an abnormally high net absorption might be expected². Finally it was predicted that just as the severity of the hypovitaminosis is influenced by the Ca-P intake, so also this would probably prove to be a controlling factor in hypervitaminosis. These suppositions have proved to be justified, and a preliminary report showing that the severity of the hypervitaminosis is governed by the calcium intake appeared a year ago [Harris, 1930].

added that Windaus now employs the minimal lethal dose as a measure of the vitamin D activity of irradiated ergosterol products [Jephcott and Bacharach, 1930]. We must point out, however, how large is the number of workers who have been led to adopt the view that toxicity is associated entirely with by-products and not vitamin D [Adam, 1928; Reyer and Walkoff, 1928; Heubner, 1929; Steudel, 1929; Simmonet and Tanret, 1929, 1930; Holtz and Brand, 1929; Haendel and Malet, 1929; Vara-Lopez, 1930; Borghi, 1929]. A later view of Dixon [1929] and Hoyle [1930], that hypervitaminosis could only be produced on synthetic diets and not on a "natural" diet such as bread and milk, is accounted for in the present paper [see also Harris, 1930], where it is shown that the exact level of overdose of irradiated ergosterol needed to produce toxic symptoms varies with the Ca/P ratio of the diet.

¹ Similarly in rickets there is a deficient calcium content of muscle [Aschenheim and Kaumheimer, 1911; Haury, 1930].

² *Net absorption* = Ca [or P] intake *minus* faecal output = absorption from gut *minus* excretion into gut. In earlier papers the alternative phrase "retention from gut" was used in order to indicate that we were taking into account this factor of a possible excretion into the gut, *i.e.* that we were dealing with the "net" and not the "gross" absorption. The use of this phrase was perhaps unfortunate, since it may have led [*e.g.* Watchorn, 1930, 1] to confusion with the quite different concept of the retention by the animal as a whole (*i.e.* intake less urinary and faecal output). Of course we nowhere postulated that the retention as a whole was increased in hypervitaminosis, in fact we were the first to draw attention to the enormously increased urinary Ca excretion.

In the present communication our experiments, begun in 1928 and concluded in the spring of 1930, are described in greater detail and discussed more especially in relation to the mode of action of the vitamin. Convincing evidence is furnished to substantiate the theory that vitamin D exerts its action by raising the blood-Ca or -phosphate, so giving rise to an increased calcification. It is shown that the increase in the calcium and phosphate is associated with an increased net absorption from the gut (this is of special consequence in hypervitaminosis with diets rich in Ca), but also that the shaft of the bone may provide an important additional source under certain circumstances, for example on low Ca diets and with larger excess of vitamin D. The theory that vitamin D might function by drawing Ca and P out of the bone was put forward by Hess, Weinstock and Rivkin [1929, 2] and is supported by work in other laboratories carried out simultaneously with our own [Light, Miller and Frey, 1929; Hess, Weinstock and Rivkin, 1930; Baumgartner, King and Page, 1929; Brown and Shohl, 1930; Weinmann, 1929] as well as by that of Watchorn [1930, 2] in this laboratory. Jones, Rapoport and Hodes [1930, 2] on the other hand have concluded that the Ca comes from the food and not the bone. It does not appear to have been realised that these two methods of calcium mobilisation may both be involved, and to varying extents dependent on the Ca-P content of the diet and the degree of excess of the vitamin.

EXPERIMENTAL RESULTS.

All the experiments to be described here have been carried out on rats; we have obtained similar results with rabbits. The strain had been inbred for many generations, and carefully matched litter mates were always evenly distributed between the several groups in each experiment. Both piebalds and albinos (the latter originating from Wistar Institute stock) were used.

The irradiated ergosterol used in the later experiments¹ had an anti-rachitic activity of approximately 10^7 (Pharmaceut. Soc.—Med. Res. Council) units per g. and in the earlier experiments of approximately one-half to one-fifth as much.

In general, the degree of severity of the hypervitaminosis in a given experiment is reflected in a proportional impairment of growth, or loss in weight, so that the growth curves presented below convey a very fair impression of the relative toxicity of the various diets.

1. Influence of variations in the calcium phosphate content of the diet.

The same basal diet (Steenbock's rachitogenic diet [Steenbock and Black, 1925] with the calcium carbonate omitted) was fed to all groups, but with varying graded additions of calcium phosphate.

It was found (Fig. 1) that each increase in the calcium phosphate allowance gave rise to an increased severity of hypervitaminosis. By sufficiently re-

¹ We are indebted to Messrs J. Nathan and Co. for generous gifts of irradiated ergosterol.

ducing the calcium phosphate a level of vitamin D excess which was otherwise definitely toxic became virtually innocuous.

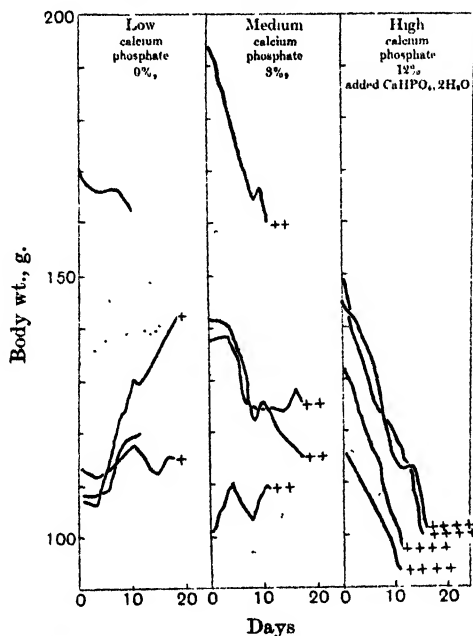


Fig. 1. Dependence of severity of hypervitaminosis D upon amount of calcium phosphate in diet.

- + Microscopic calcium deposits (in kidney only).
- + + Definite calcareous deposits.
- + + + Very extensive calcareous deposits.
- 10 mg. irradiated ergosterol/rat/day.
- Controls without vitamin D excess.

2. Comparison of natural and synthetic diets with varying content of calcium phosphate.

A series of tests was carried out to determine quantitatively the comparative effects of diets of Hovis bread and milk on the one hand, and synthetic diets, rich in calcium and phosphate, on the other, when fed in conjunction with various graded excesses of vitamin D. The results show that doses which are just on the border line of toxicity for the synthetic, salt-rich diet may be practically harmless for the bread and milk diets. However, when slightly larger excesses of vitamin D are given the difference becomes less noticeable, and with still larger excesses the difference virtually vanishes (Fig. 2). Next it was shown that the addition of an inorganic salt mixture (consisting principally of calcium salts and phosphates [Harris and Moore, 1928]) to the bread and milk diet sufficed to render it indistinguishable from a synthetic diet, in its power to permit hypervitaminosis at a given level of vitamin D excess (Fig. 3). Finally, we were able to demonstrate that on a diet consisting wholly of dried milk, and therefore very rich in Ca, an even more severe hyper-

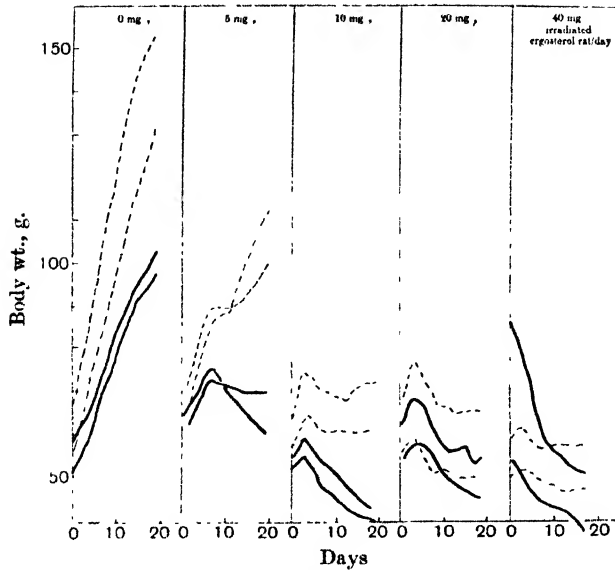


Fig. 2. Relative toxicity of vitamin D excess, on bread and milk and synthetic diets.

--- Hovis bread and milk (50:50).
— Synthetic diet.

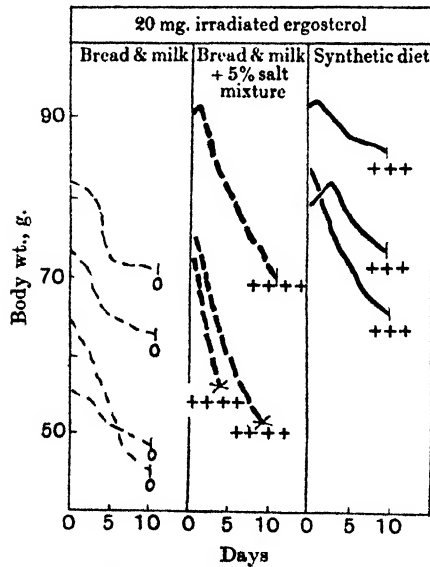


Fig. 3. Bread and milk diet *plus* added salt mixture permitting hypervitaminosis as readily as synthetic diet.

x Animal died. | Animal killed.
0 No calcaeous deposits yet appeared.
+ + + Considerable calcaeous deposits.
+ + + + Most extensive calcaeous deposits.

vitaminosis was produced than on the usual synthetic diet, at the various graded levels of vitamin excess¹ (Fig. 4).

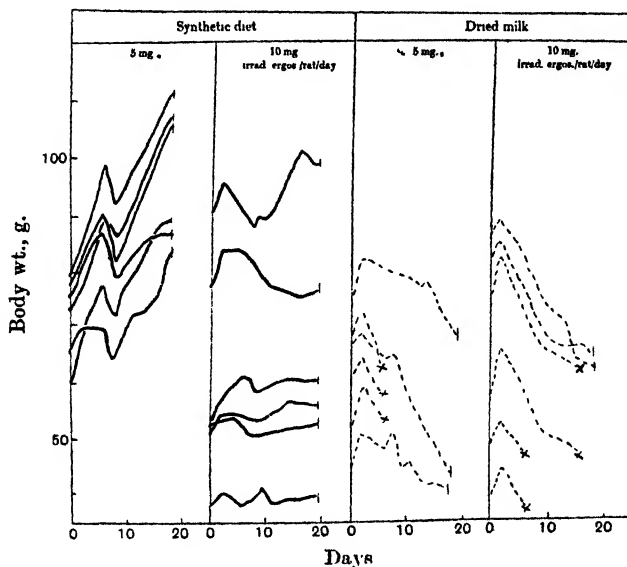


Fig. 4. Sole diet of milk even more favourable for production of hypervitaminosis than synthetic diet (4–5% salts).

× Animal died.

| Animal killed.

3. Influence of variations in Ca/P ratio.

These experiments were planned with the object of comparing the effects of large doses of irradiated ergosterol when administered in conjunction with

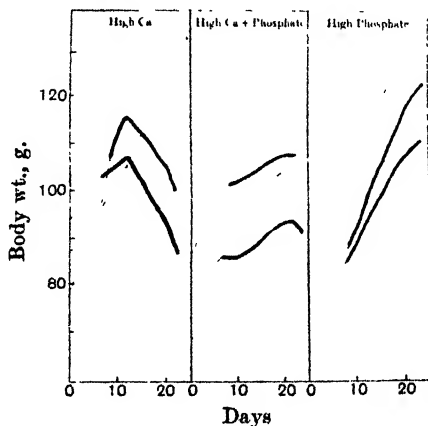


Fig. 5. Increased toxicity of irradiated ergosterol with increased Ca/P ratio of diet.

— Irradiated ergosterol (0.05% of diet).

..... Controls without vitamin D excess (1 drop "radiostol" per day).

¹ These results afford an explanation of the results of Dixon [1929] but fail to support his main conclusion:—"the same experiments made on animals living on an ordinary diet show that excess of the 'poisonous' food is harmless."

diets (1) rich in calcium but deficient in phosphate (2) rich and well-balanced with respect to both calcium and phosphate, and (3) deficient in calcium but rich in phosphate. The same basal diet was used throughout (based on Steenbock's formula [Steenbock and Black, 1925]: yellow corn, 60 g., gluten flour, 200 g., sodium chloride, 10 g.); but while the calcium-rich diet contained 38 g. of calcium lactate, the calcium- and phosphate-rich diet contained 30 g. of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ and the high-phosphate, low-calcium diet contained 24.8 g. of Na_2HPO_4 .

Our results (Fig. 5) showed that a given overdose of irradiated ergosterol, just sufficient to cause rapid loss of weight with diets rich in calcium and deficient in phosphate, loses much of its toxicity when the calcium is balanced by the addition of phosphate, and that it becomes relatively harmless when the calcium is omitted and the phosphate remains high. This held true even when the feeding was prolonged for many weeks (Fig. 6). In further experi-

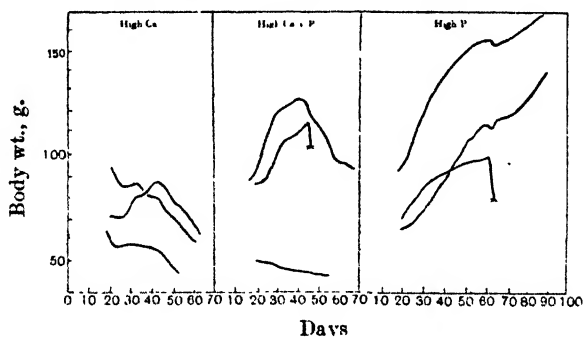


Fig. 6. Long-time feeding experiments, comparing high-calcium, high-phosphate, and high-calcium-plus-phosphate diets.

— Irradiated ergosterol. Controls.

ments (Fig. 7; cf. Fig. 8) in which the ergosterol was administered at higher levels or more fully-grown animals were used, a similar difference between effects of the high-calcium and the high-phosphate diets could still be detected.

Measurements of the calcium and phosphate metabolism of these animals are reported separately [Watchorn, 1930, 2]. The *post mortem* abnormalities characteristic for each group are discussed in Sections 6 and 7.

4. Production of a distinctive type of hypervitaminosis on calcium-free or calcium- and phosphate-free diets.

Since it had been shown (Figs. 5, 6, 7) that normal growth was permitted on high-P, low-Ca diets with such levels of irradiated ergosterol as were sufficient to cause rapid loss in weight and death when fed with diets containing both calcium and phosphate (or high-Ca, low-P), it was decided to ascertain whether even larger doses of irradiated ergosterol would still prove harmless under these conditions.

The results show that a toxic effect can be produced (at least eight times

as much irradiated ergosterol being needed when fed with a high-P, Ca-free diet as with a normal Ca-P diet, in order to produce loss in weight at an equal rate (Fig. 8)), but that there is a striking difference in the nature and

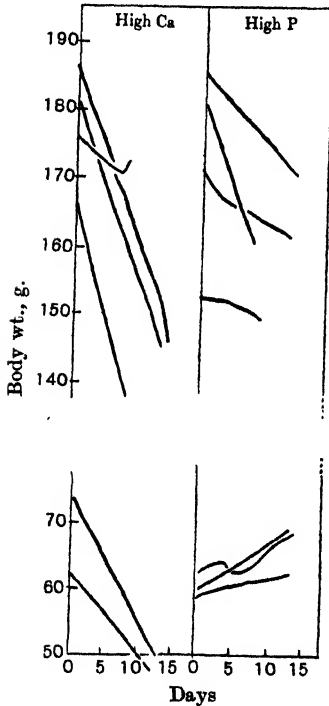


Fig. 7.

Fig. 7. Comparison of high-calcium and high-phosphate diets at higher level of vitamin D excess. Adults shown above. Representative curves from 50 animals.

Fig. 8. Hypervitaminosis induced on high-P, low-Ca diet by sufficiently increased vitamin D intake. — Ca-deficient diet. — Control diet, normal Ca and P. × Animal died.

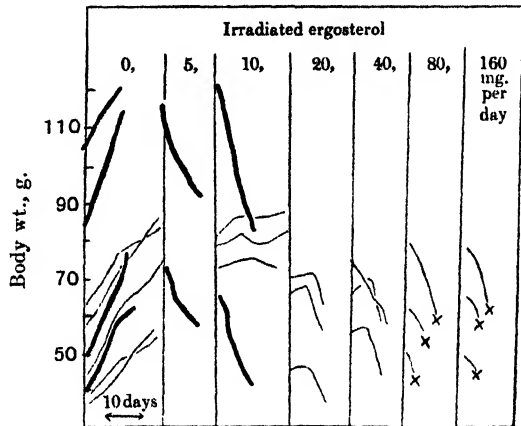


Fig. 8.

symptoms of the hypervitaminosis in the two cases. Whereas the controls, on the diet containing both calcium and phosphate and 10 mg. irradiated ergosterol, showed the classical picture of hypervitaminosis, with calcification of vessels, heart and kidney, stimulation of bone formation, and, in the early stages, absence of noticeable osteoporosis in the cortex of the bone shaft or in the jaw bone, the experimental animals on the calcium-free diet with vitamin excess showed a rickety appearance in the bones, a pronounced resorption of compact bone together with almost complete absence of the usual calcification of vessels, kidneys, etc. In short, the changes in hypervitaminosis on the Ca-free diet resemble those on the control Ca-free diet without any vitamin excess, except that loss of mineral substance from the spongiosa is more extensive¹.

¹ The high-phosphate diet *per se* always tends to give rise to a characteristic kidney lesion, entailing focal necrosis of the tubules of the boundary zone, both in the hypervitaminosis and in the control groups.

Diets deficient in Ca and P. On a diet deficient in both calcium and phosphate, similar results were obtained (Fig. 9). Loss of weight and death resulted if sufficiently augmented doses of irradiated ergosterol were given, but

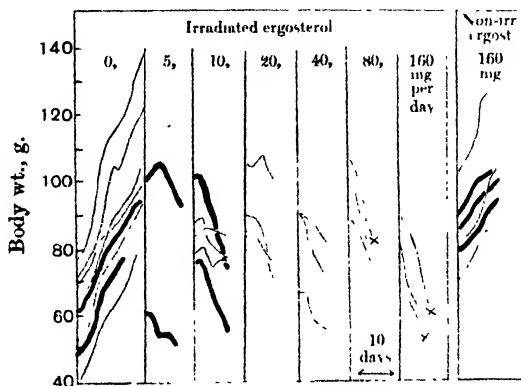


Fig. 9. Hypervitaminosis produced on diets deficient in Ca and phosphate.
 — Ca- and P-deficient diet. — Control diet, normal Ca and P. x Animal died.

no appreciable deposits of calcium salts had appeared in the organs, vessels or bone-endings, while the bones were soft and fragile with marked evidence of resorption and a rickets-like beading at the costo-chondral junction (again resembling the controls in the Ca- and P-deficient group without the vitamin D excess but with hastened resorption of spongiosa).

5. Radiographs of bones.

In view of the well-known effects upon bone structure of deficiency of vitamin D or of abnormal Ca-P intake, a detailed X-ray study was made of animals receiving excessive vitamin D, in the various groups and sub-groups already described¹. The typical effects of hypervitaminosis upon the bony skeleton were best seen at the growing end of the long bone, and with diets containing adequacy of both Ca and P in normal ratio. Again the picture in hypervitaminosis may be contrasted with that in rickets (Plate I). In the early stage of hypervitaminosis, or when only moderate overdoses were given, an unmistakable narrowing of the metaphyseal cartilage was always seen. The epiphyseal end of the diaphysis became highly calcified, forming a characteristic dense band which rapidly broadened and extended to the marrow cavity. When the irradiated ergosterol was fed at very high levels of toxicity and the observations were continued for a sufficiently long period until the animal had lost weight considerably, it was found that the dense area continued to recede into the marrow cavity but left behind a distinct gap of lessened density, at the site which would normally correspond with the growing end of the bone.

¹ The exposures were made in the University Radiological Department, under the general supervision of Dr A. E. Barclay.

In contrast with the foregoing we found that when equally large overdoses of vitamin D were fed, but in conjunction with a diet such as is normally able to retard calcification (*e.g.* Steenbock's rachitic ration), excessive calcification of the bone-ending was in fact much less evident, or absent, although the production of calcareous deposits in the soft tissues (see below) was appreciably intensified.

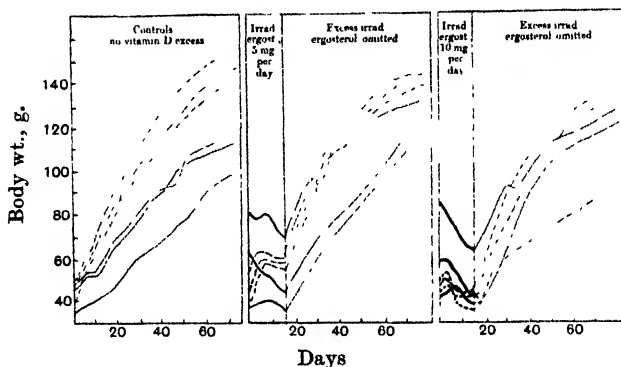


Fig. 10. Hypervitaminosis and cure (normal synthetic diet, and Steenbock rachitogenic diet).

Broken line, normal diet.

Continuous line, Steenbock diet.

Thickened line denotes excess of vitamin D.

6. Factors favouring the formation of the calcareous deposits.

An examination of our *post mortem* records for the various experiments described above makes it clear that the formation of the calcium deposits in the soft tissues (kidneys, arteries, non-striated muscle, *etc.*) is greatly increased when there is an abundance of calcium salts in the diet. As will be shown below the ingestion of large doses of vitamin D under such conditions gives rise to a greatly intensified hypercalcaemia (or hyperphosphataemia), associated with an increased net absorption from the gut. Examples of the influence of the calcium content of the diet in inducing calcareous depositions are seen in the following instances: (1) animals receiving Hovis bread and milk with 20 mg. of irradiated ergosterol lost weight rapidly but showed no calcium deposits after 10 days, whereas animals receiving the same diet *plus* 5 % added salt mixture had extreme and extensive calcium deposition, and animals on synthetic diet containing 5 % salt mixture showed fairly severe calcification; (2) after 12 days on 10 mg. of irradiated ergosterol and synthetic diet there were no naked-eye calcium deposits in animals given the low calcium phosphate diet, but there were definite calcium deposits in animals given medium calcium phosphate, and very extensive calcium deposits in animals given high calcium phosphate; (3) there was a virtual absence of calcareous deposits in animals having excess vitamin D with diets devoid of calcium, or devoid of calcium and phosphate (Figs. 8, 9); and (4) the calcium deposits were more readily produced on a diet of dried milk (Fig. 4) than on a diet

of bread and milk (Fig. 3). With the low-calcium diets, as will be shown, a relatively greater amount of calcium is drawn into the blood from the bone.

Two other factors concerned are the duration of the hypervitaminosis and the degree of excess of vitamin D in the diet. With insufficient excess, no calcium deposits are seen; with very large excess, the animal may die before the deposits have had time to develop. An animal may lose weight with hypervitaminosis for 15 days or longer before showing calcareous deposits (*e.g.* on the various diets referred to in Fig. 5; bread and milk, Fig. 3; etc.). Hence, the most favourable condition for the development of the calcareous deposits is a long-drawn-out hypervitaminosis, with no greater excess of vitamin D than is needed to cause a slow loss in weight, and an abundant supply of calcium salts in the diet¹.

7. *Histology of bone and teeth; osteogenesis and resorption in hypervitaminosis.*

The abnormalities seen in the bony skeleton² in hypervitaminosis D vary somewhat according to the stage of development, and also with the mineral salt content of the basal diet.

The effects seen with normal basal diets (complete synthetic diet; bread and milk; milk; *etc.*) may be described first. In the *teeth*, the most noteworthy feature is a remarkable overgrowth of cement, which extends to three or four times its normal thickness, shows much cellular proliferation and invades the marrow spaces of the jaw bone. The inner part of the dentine, to the extent of perhaps half its transverse thickness, is transformed into a distinctive type of secondary dentine, possessing a roughly granular and irregular laminated structure and staining intensely with haematoxylin (Plate II A).

In the *long bones* (or *ribs*) (Plate II B) the first change, observed with moderate over-doses of the vitamin and before toxic symptoms have appeared, is a marked stimulation of new bone formation. Osteogenesis is very active, and abnormally long and slender trabeculae are produced, extending far into the marrow cavity and already fusing to some extent. This is in conformity with the early radiograms (Section 5), which shows a dense shadow at the sub-epiphyseal region. At this stage the bones are solid and compact and there is little evidence of loss in density in the cortex of the shaft of the long bones or in the jaw bone. This is the picture seen in rats receiving bread and milk *plus* 5–10 mg. of irradiated ergosterol after 27 days (Fig. 2), or

¹ The contention that the calcium deposition in hypervitaminosis is subsequent to necrosis of the tissues is discussed elsewhere [Innes, 1930, *Ann. Rep. Dept. Animal Path., Camb.* (in

² The specimens examined embrace (a) transverse sections of the lower jaw across the level of the root of the incisor teeth, (b) longitudinal sections of the costochondral junctions, and (c) longitudinal sections through the distal end of the femur and the proximal end of the tibia. These were fixed in formaldehyde saline and decalcified in 7% HNO₃, embedded in paraffin in the usual way, and stained with haematoxylin and eosin.

synthetic diet *plus* 5 mg. irradiated ergosterol after 10 days (Figs. 8 and 9)¹. As the process advances, the fusion of trabeculae continues so that a great mass of spongy bone is formed, which extends some considerable distance up the marrow cavity and occupies its entire width (Plate II B, no. 2). In the advanced degree of hypervitaminosis (Plate II B, no. 3), however, the most prominent feature is the evidence of excessive resorption. Macroscopically the bones become soft and fragile. The cortex of the shaft of the long bones and the jaw bone also, instead of remaining compact, is extensively osteoporosed and osteoclastic activity is prominent. Similarly a large marrow space appears at the epiphyseal end of the abnormal mass of spongiosa. This finding is in agreement with the radiograms taken during the late stages of hypervitaminosis, in which the dense shadow appears to have receded from the epiphysis. The picture here is complicated by the cessation of bony growth. The intermediate growth cartilage is very narrow and lined on the diaphyseal side by a thin transverse plate of bone. (This account applies, for example, to rats receiving bread and milk and the higher levels of irradiated ergosterol.)

The resorption from bone is much lessened when the diet is rich in calcium. Thus on a high-calcium, low-phosphate diet (Fig. 6), the animal may die with extensive calcareous deposits, but the bones and teeth will show no osteoporosis or other abnormalities, except those due to cessation of growth. On low-calcium diets, on the other hand, the resorptive changes become intensified. For example, the cortex is less compact on a bread and milk diet with hypervitaminosis than it is on a Ca-rich synthetic diet with the same excess of vitamin D (Fig. 2). Finally on Ca-free diets, or diets rich in phosphate and deficient in calcium, resorption is very extensive. A rickets-like appearance is seen resembling that in the controls on the same diets without the excessive vitamin, but in addition there is also a disappearance of the sub-epiphyseal spongiosa. Thus we have evidence of the bones acting as the main source of the extra calcium drawn into the blood stream when there is an insufficient supply from the gut.

8. p_H of faeces.

It has been shown that on certain rachitogenic basal diets the faeces of the rat tend to become abnormally alkaline when there is a deficiency of vitamin D, and to revert to a more normal reaction when the vitamin is restored to the diet [Zucker and Matzner, 1923; Jephcott and Bacharach, 1926, 1928; Bacharach and Jephcott, 1929; Grayzel and Miller, 1928]. An acid reaction in the gut favours solubility of Ca and hence ease of absorption. By analogy it seemed possible that feeding of excessive vitamin D might give rise to faeces with an abnormally low p_H figure. It has already been reported [Harris and Moore, 1929, 1] that, on an ordinary synthetic diet, no very decisive increase in acidity is in fact obtained. In continuation, we have examined the effects of excessive vitamin D given in conjunction with a

¹ These changes were not seen in adult animals where bony growth has ceased, but the formation of calcareous deposits was very marked.

Zucker diet [Jephcott and Bacharach, 1926], such as readily permits the change of faecal p_H in vitamin D deficiency¹. Here again a similar result was found (Fig. 11).

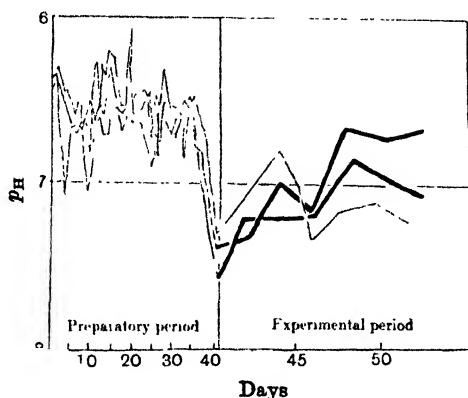


Fig. 11. p_H of faeces in hypervitaminosis.

— Excessive vitamin D.
 - - - Control, or preparatory, diet (low in, or devoid of, vitamin D).

9. "Vitamin balance."

In earlier papers [Harris and Moore, 1928; 1929, 1] we showed that the degree of severity of the symptoms of hypervitaminosis was sometimes diminished by an increase in the vitamin B (or B + C) allowance. This result has been fully confirmed [*e.g.* Duguid, Duggan and Gough, 1930].

A somewhat similar phenomenon was the production of symptoms resembling avitaminosis B by feeding excessive cod-liver oil [Harris and Moore, 1928] or cod-liver oil concentrate [Harris and Moore, 1929, 2]. These symptoms could be cured or prevented by a slight increase in the vitamin B allowance. The same result has since been obtained independently by American workers [Norris and Church, 1930]. However, we thought it necessary to emphasise that this balancing effect could only be readily seen when the vitamin B allowance was already low, and the cod-liver oil excess very considerable.

Further work on hypervitaminosis D confirms the view that similar conditions apply here. If the diet is already rich in vitamin B, further small additions fail to show any appreciable alleviating effect in protecting the animal against massive overdoses of vitamin D (Fig. 12). It is clear therefore that in interpreting such results, a limited significance only can be attached to the conception of a balance between vitamins D and B.

DISCUSSION.

The observations recorded in the above experimental section, taken in conjunction with results described in earlier papers, appear to furnish us with

¹ The electrometric determinations of p_H were kindly carried out by Miss E. Allchorne of the Glaxo laboratory.

a logical and consistent picture of an orderly sequence of changes induced by the action of vitamin D in the animal organism. As we pass from a deficient intake of vitamin D to an adequate level, then to an abundance, and so to excess, and finally to very great excess, a series of phenomena is seen in increasing intensity. Our main deductions may first be summarised briefly as follows, and we can then proceed to a more detailed critical analysis. An increased vitamin D intake promotes increased absorption of Ca and/or phosphate from the gut (or diminished excretion into the gut), so tending to raise the level in the blood. With inadequacy of vitamin D, the blood fails to

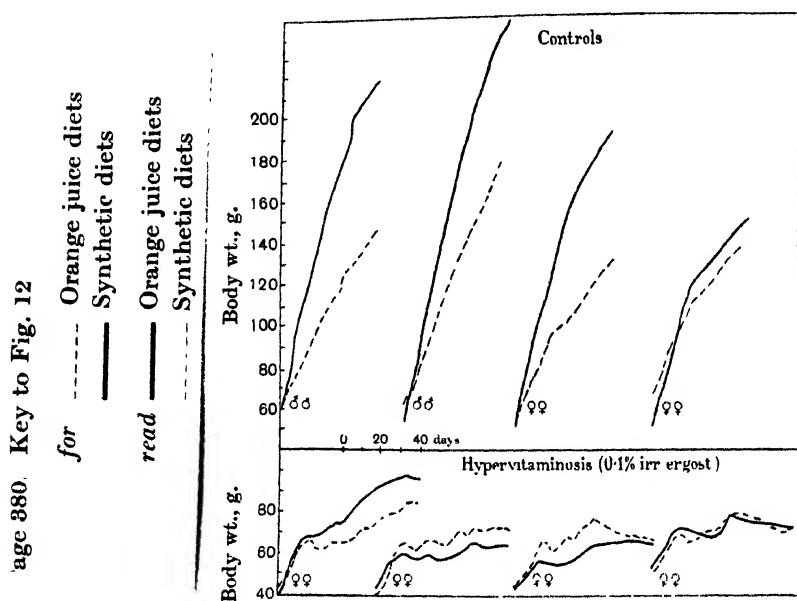


Fig. 12. Hypervitaminosis on (1) synthetic diet, and (2) with the sugar (60% of diet) replaced by equivalent of concentrated orange juice (note remarkable growth rates of controls on the orange juice regimen).

secure sufficient Ca and/or phosphate; with moderate amounts the blood succeeds in maintaining its approximate constancy of composition; while with large overdoses, hypercalcaemia and/or hyperphosphataemia cannot be averted. In the latter condition the kidney responds by an abnormal urinary excretion of Ca and/or P. If on the other hand the excessive vitamin D be now suddenly removed, a suitably increased excretion of Ca and phosphate is permitted to the gut [Watchorn, 1930, 1]. The "retention" of Ca or phosphate by the animal as a whole is seen to be the resultant of two factors, working in opposite directions, *viz.* (i) the increased absorption from (or diminished excretion into) the gut, and (ii) the increased excretion by the kidney. As more and more vitamin D is given the latter factor overtakes the former, so that the resulting retention, while it is increased by moderate doses

of the vitamin, is diminished by very large doses. The deposition of calcium salts in certain sites is associated with a withdrawal from others; in hypervitaminosis this transference process in the bone is apt to be excessive; in rickets there is evidence of a defective transfer [Morelle, 1930]. As has been shown in the experimental section, when the diet is deficient in calcium the withdrawal from the bone stores becomes the noteworthy feature of the hypervitaminosis. When on the other hand the diet is rich in calcium the bone is less called upon, but there is increased liability to calcareous deposition as a result of an increased net absorption from the gut, and partly also no doubt because the amount stored by the bone is less than normal. Each addition of calcium to the diet intensifies the hypercalcaemia and calcareous deposition with a given overdose of vitamin D.

It is clear, therefore, that the precise level of Ca and P in the blood as influenced by the vitamin D intake depends on a greater variety of complex factors than has been generally realised in the past, including gut absorption (or excretion), kidney excretion, and deposition and dissolution of calcium deposits.

The experimental results are not inconsistent with the simple view that vitamin D acts by increasing the apparent solubility of the Ca and phosphate in the blood, an increased content in the blood facilitating deposition in the sites to be calcified. The latter assumption seems justified in view of the observation [Shipley, 1924; Shipley, Kramer and Howland, 1926] that non-calcified cartilage from a rachitic rat will rapidly calcify once it is put in blood-serum or inorganic saline containing adequate Ca and P (or $\text{Ca} \times \text{P}$ product). Our evidence also indicates that vitamin D can serve to promote osteogenesis. This could be explained readily enough, however, by supposing that the rate of formation of fresh osteoid tissue depends on the rate at which the old becomes calcified, in which case clearly the precipitation of extra calcium would mechanically serve to stimulate fresh osteogenesis.

It is obvious that much information has still to be acquired before a full explanation can be given for the preferential deposition of calcium salts in certain types of tissue to the total exclusion of others; this is seen as well in hypervitaminosis as under normal physiological conditions. The presence of phosphatase [Martland and Robison, 1924] is doubtless of special importance, so too perhaps the alkalinity.

The suggestion that vitamin D mobilises Ca (or P) by stimulating the parathyroid demands consideration. Obviously the theory could be tested by determining the influence of vitamin D after parathyroidectomy. The results of such tests hitherto recorded are very conflicting [Hess, Weinstock and Rivkin, 1929, 1, 1930; Jones, Rapoport and Hodes, 1930, 1; Taylor, Branion and Kay, 1930]. Nevertheless it seems probable that vitamin D can function independently of the parathyroid, for the parathyroid hormone raises the calcium in the blood solely by withdrawing it from the bones and without increasing the net absorption from the gut.

Certain points in the main conclusions outlined above appear to run counter to current views or to recent observations by other workers, and the evidence in their favour will now be analysed in greater detail.

Gut absorption.

The measurements taken by Miss Watchorn of calcium and phosphate intakes and faecal outputs upon the animals dealt with in Fig. 7 (Steenbock basal diet, (1) with added calcium and no phosphate, and (2) with added phosphate and no calcium) appear at first sight to contradict our assumption of an increased stimulation of gut absorption through the agency of vitamin D. However, in interpreting her results [Watchorn, 1930, 2], several complicating factors have to be borne in mind, most notably that of starvation, or general failure. Once a severe degree of hypervitaminosis has supervened (so that the animal is consuming only a very small fraction of its normal food intake and body processes are failing and perhaps calcium deposits already appearing in kidneys, muscles, vessels, intestinal tract, *etc.*) the gut function appears to break down and the effect of vitamin D in increasing the absorption can now no longer be demonstrated. This was illustrated clearly enough in Watchorn's earlier paper [1930, 1], in which she showed, working with normal basal diets, that vitamin D excess at first caused an increased net absorption (in every instance but one), particularly when the absorption had previously been low, but that this absorption ultimately fell off, as the animal ceased to eat. Partial starvation, in fact, was shown in a control experiment to be sufficient in itself to diminish Ca and P absorption. If now we review in the light of these considerations the results with the Steenbock high-Ca diet [Watchorn, 1930, 2], we find that the analyses refer to the period when these very complications had already set in and the animals were losing weight and taking very little food (in the second week of hypervitaminosis). An unequivocal demonstration of increased net absorption could not therefore be expected. Even so, it can be shown that some effect on the Ca is still apparent, when figures are recalculated upon a percentage basis, in the case of two animals out of the three. The third animal may be fairly neglected for its food intake had dropped 75 %, compared with only 46 % and 54 % for the other two, and absorption from the gut had indeed virtually ceased.

The phosphorus absorption in this test showed immense fluctuations between the three animals in the group, varying from 56 to 77 % in the preliminary period, from 64 to 15 % in the hypervitaminosis period, and from 57 to 15 % in the recovery period¹. However, a similar investigation has been carried out by Brown and Shohl [1930], but embracing a series of different levels of vitamin D feeding; and since their results have the advantage of showing greater uniformity than those just discussed, it has been thought

¹ This relates to the organically bound P of the phosphate-free Steenbock diet. Since in vitamin D metabolism we are primarily concerned with inorganic phosphate, the meaning of the figures might conceivably be disputed, but the results of Brown and Shohl (*v. infra*) definitely show its increased absorption under the influence of vitamin D.

worth while to recalculate their figures, also upon a percentage basis, in order to demonstrate the remarkable consistency and regularity with which each increase in vitamin D does in fact give rise to an increased net absorption from the gut, until finally the toxic complications supervene. Thus on Steenbock's rachitogenic diet (high-calcium, low-phosphate) the addition of 10,000 times the minimal effective dose of vitamin D increased the net absorption of phosphorus by no less than 300 %, and of Ca by about 50 %. With still larger doses, and the appearance of toxic symptoms and loss of appetite, the absorption begins to fail (Table I). Brown and Shohl themselves, although drawing attention to the increased urinary elimination of Ca and P with large doses of vitamin D (the excretion as they say shifting from the faeces to the urine, and the retention decreasing), did not attach special significance to the question of gut absorption, in fact they stated that the amount of Ca and P excreted remained relatively constant (in the absence of toxic symptoms).

Table I. *Increase in net absorption of Ca and P resulting from increasing doses of irradiated ergosterol. Recalculated from Brown and Shohl [1930]. (Steenbock high-Ca, low-P diet).*

Daily dose of irradiated ergosterol (mg.)	0	0.01	0.1	0.5	1.0	2.0
P "absorbed," % of intake	8, 12	22, 27	26, 26	33, 37	41, (21)*	(27)*
Ca "absorbed," % of intake	31, 36	42, 43	43, 49	48, 48	49, (34)*	(34)*

* Marked loss of appetite.

A similar examination of Brown and Shohl's figures for a diet (Sherman) with a normal Ca/P ratio, as opposed to the rachitogenic, high-Ca, diet, shows a corresponding if less intense effect. An increase of vitamin D by about 2000 times above the minimal protective dose gives rise to a well-marked increase in the weight of Ca and P absorbed (Fig. 13), while at higher levels the complicating influence of the lowered food intake is again evident, even when results are calculated on a percentage basis (Fig. 14).

With our diet rich in phosphate but free from Ca (Fig. 7) the net absorption of P was already at so high a level (83 to 93 %) in the control that the demonstration of any increase under the action of vitamin D was not to be anticipated. It may be recalled indeed that at the level of vitamin D excess employed no toxic ill-effects were seen with this diet. As has already been suggested the effect on phosphate may be secondary to that on Ca.

But perhaps the most striking argument that can be deduced in support of our view, that large doses of vitamin D tend to raise the absorption of Ca and phosphate (or to diminish their back excretion into the gut), is obtained from a study of the effects of a cure in hypervitaminosis. An animal having the typical abnormally high blood-phosphate or -Ca induced by excessive doses of vitamin D is suddenly put back on to a diet free of any such excess. The curative process begins immediately. The absence of vitamin D, it can

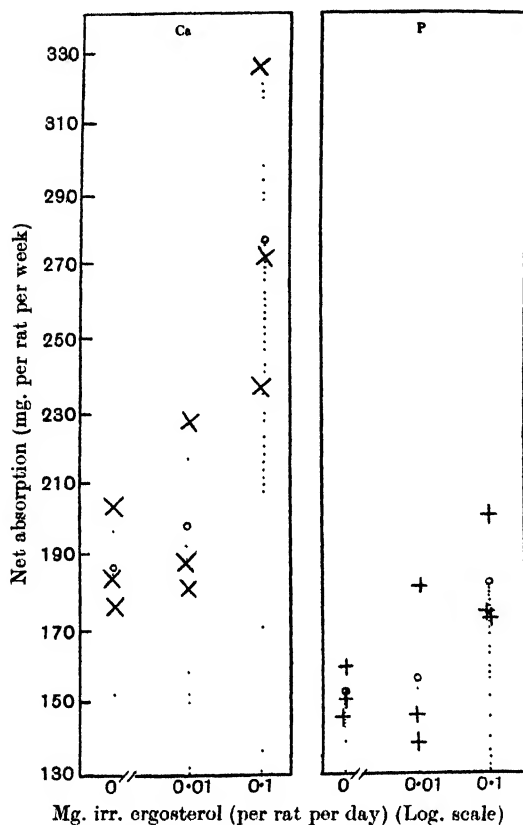


Fig. 13. Increase in Ca and P net absorption with increasing doses of irradiated ergosterol. Recalculated from Brown and Shohl. Normal diet (Sherman).
0 = mean readings.

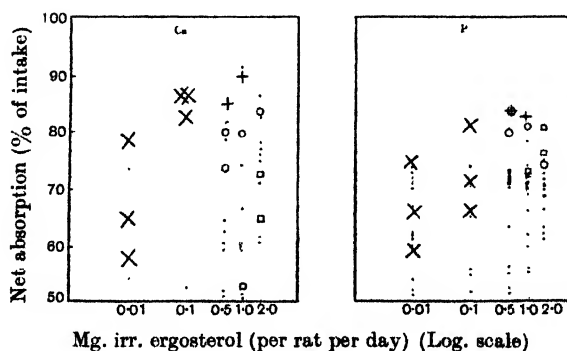


Fig. 14. Complicating influence of failure and loss of appetite with high levels of toxicity.

X Intake approx. normal, 240–390 mg. Ca per week.
+ „ below normal, 170 „
o „ low, 110–150 „
■ „ very low, under 100 „

be shown, now permits the animal to excrete in its faeces a vastly increased amount of Ca or P, more suitable to the excessive blood level and tending to reduce it towards the normal. The faecal calcium now reaches figures as high as 65–90 %, compared with only 10–20 % in the presence of the excessive vitamin D, or 20–60 % for the appropriate controls (expressed as percentage of intake) (Fig. 15). Thus the point of view we are here advocating will allow

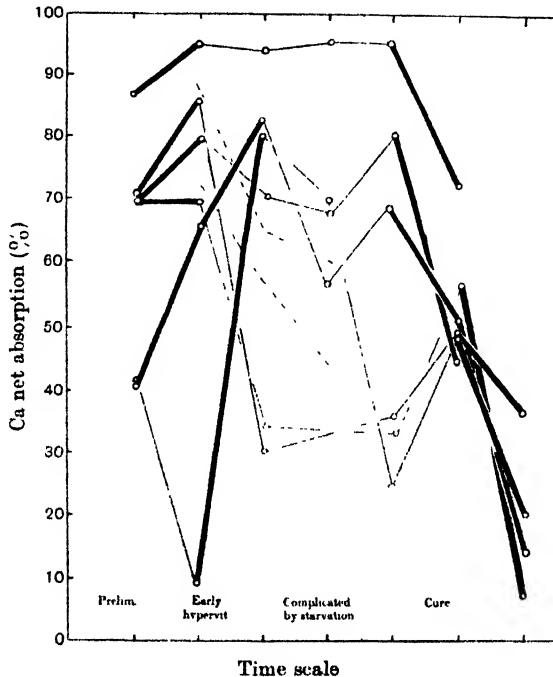


Fig. 15. Rise in net absorption at beginning of hypervitaminosis, complicating influence of starvation, and fall in cure.

— — — Starvation control without hypervitaminosis.

a rational interpretation of the underlying causes of the “even graver disturbances in absorption and retention during the recovery period,” to which attention was first drawn by Watchorn [1930, 1].

We have therefore the following evidence pointing to increased net absorption under the influence of vitamin D:

(1) the dependence of the hypercalcaemia and calcareous deposition in hypervitaminosis D upon the Ca intake;

(2) the occurrence of a greatly increased faecal excretion when the hypervitaminosis is terminated;

(3) direct calculations upon data for intakes and faecal outputs, including the earlier uncomplicated stages of hypervitaminosis;

(4) measurements by earlier workers upon clinical and experimental hypovitaminosis and its cure.

Raised blood-Ca and/or -phosphate.

The production of hypercalcaemia and/or hyperphosphataemia in rabbits or rats by the action of excessive doses of vitamin D, first reported by Harris and Stewart [1929], and repeated under a wider variety of conditions [Harris, 1930], has been repeatedly confirmed, *e.g.* by Ashford [1930] who showed that there was no concurrent increase in the organic acid-soluble phosphate, and the observation has been extended to other species including dogs [Jones, Rapoport and Hodes, 1930, 1], chickens [Massengale and Nussmeier, 1930], and cows [Greig, 1930]. But certain workers have obtained less consistent or even negative results, the reasons for which demand consideration here.

In the first place the important consideration must be borne in mind, as originally indicated [Harris and Stewart, 1929] and again confirmed in our present results, that the severity of the condition is largely governed by the amounts of calcium or phosphate ingested. The influence of this factor is clearly apparent also in the recent result of Warkany [1928], who showed that the rise in blood-phosphate following a meal of inorganic phosphate is greatly increased by the administration of irradiated ergosterol. In the second place it must be realised that an increase in both Ca and P simultaneously is not necessarily seen; sometimes one is affected first, sometimes the other. The influence of calcium equilibrium upon phosphate and *vice versa* is apparent here. Next, the hyperphosphataemia or hypercalcaemia, varying as it does with the diet, is to be understood in a relative rather than an absolute sense; for example, the figures of Shohl, Goldblatt and Brown [1930] show that in rats fed on high-phosphate or high-calcium diets with excess vitamin D, the blood-Ca or -P is high compared with the corresponding controls, although not necessarily of course compared with the normal rat. Finally, negative results have sometimes undoubtedly resulted from failure to give sufficiently large excess of the vitamin. It was earlier suggested [Harris and Moore, 1929, 1, p. 272] that such an explanation might account for the observations of Havard and Hoyle [1928], who, working on man, were unable to obtain any rise in inorganic P or serum-Ca above the low winter level. Confirmation for this view appears to be found in more recently published work on human beings, by various authors, in which very high levels of vitamin D have been fed, and one or other of the following typical abnormalities reported, *viz.* hypercalcaemia, or hyperphosphataemia, or increased renal excretion of P or Ca, or overcalcification of bone-endings, and, even, calcareous deposits [Hess, Lewis and Rivkin, 1928; György, 1929; Puschler, 1929; Hughes *et al.*, 1929; Hottinger, 1929; Kroetz, 1927; Lasch, 1928; Ghirardi, 1929].

Withdrawal of calcium from bone.

Our results show that with diets very rich in calcium a relatively small excess of vitamin D is sufficient to cause hypercalcaemia (or hyperphosphataemia) and the appearance of calcareous deposits. Under these circum-

stances there is, of course, little evidence of osteoporosis. Similarly, chemical analysis showed that there was no appreciable change in the mineral content of the bone. These results were published in a preliminary communication which stressed the importance of the Ca intake as a determining factor in hypervitaminosis; but unfortunately the following statement, couched in far too sweeping terms, was made, "we find by chemical analysis that there is no loss of Ca or P from the bones to account for the rise in the blood figure" [Harris, 1930]. It should have been made clear that this related only to these particular diets. With very high doses of vitamin D, particularly on Ca-deficient diets, the withdrawal of mineral from the bone becomes the outstanding feature. (This conclusion was also reached indirectly [Watchorn, 1930, 2] from analyses of faeces and urine for the animals shown in Fig. 8.) The evidence of a similar withdrawal and re-deposition in the cure of rickets has already been alluded to.

Abnormalities in bone structure in hypervitaminosis.

In marked contrast with our evidence of immense stimulation of spongiosa formation and resorption of compact bone. Shohl, Goldblatt and Brown [1930] found "no striking abnormalities" histologically in the ribs in hypervitaminosis. On the other hand in the recent independent work of several other investigators [Kreitmair and Hintzelmann, 1928; Baumgartner, King and Page, 1929; Weinmann, 1929; Collazo, Varela and Rubino, 1928, 1929, 1, 2, 3] many conclusions may be found in common with our own. Hypercalcification of the growing end of the bone was also clearly shown in radiological observations on children by Hess, Lewis and Rivkin [1928], and on rats by Shohl, Goldblatt and Brown [1930], and by György [1929].

Urinary calcium (or phosphate) excretion.

Harris and Stewart [1929] found that in hypervitaminosis D the renal excretion of calcium was so much increased that the urine became cloudy with calcium salts. They interpreted this to mean merely that the organism was endeavouring to dispose of the excessive calcium carried into the blood stream. More recent quantitative tests have abundantly confirmed the existence of the remarkably high urinary output either of phosphate or of calcium or both [e.g. Ashford, 1930; Watchorn, 1930, 1, 2; Brown and Shohl, 1930] and the negative result of Hoyle and Buckland [1929] must presumably be ascribed to the use of insufficient excess of vitamin.

SUMMARY.

A study has been made of the influence of variations in the calcium and phosphate intake upon the abnormalities resulting from overdoses of irradiated ergosterol. An increase in the calcium content of a diet (or in the Ca/P ratio) intensifies the severity of the hypervitaminosis and gives rise to

an increased formation of the calcareous deposits, at a given level of vitamin D excess. With diets virtually devoid of calcium and phosphate, on the other hand, a hypervitaminosis of a distinctive character can still be produced provided now that the level of vitamin D excess is sufficiently raised; under these conditions calcareous deposits are not in evidence but there is a greatly increased resorption of bone substance.

X-ray and histological examinations of the bones have shown that large doses of vitamin D (with normal diets) stimulate osteogenesis, and a densely calcified overgrowth appears at the growing end of the bone (in contrast with rickets); while in the advanced degrees of the hypervitaminosis resorption is extensive, and the cortex of the shaft and other "compact" bone becomes spongy. Vitamin D excess also gives rise to a remarkable overgrowth of cement in the growing animal.

In explanation of these and earlier results it is shown that feeding of excess of vitamin D (in the various types of hypervitaminosis examined) gives rise to a raised blood-calcium or -phosphate or both, with a tendency to deposition of calcium in certain sites, just as vitamin D deficiency (*e.g.* in rickets) gives rise to lowered blood-phosphate or -calcium or both and accompanying inadequate calcification. The extra calcium and phosphate may be derived in two ways: (1) increased net absorption from the gut; and (2) increased withdrawal from bony stores, vitamin D therefore having a distributive action. In the hypervitaminosis produced on diets rich in calcium with moderate overdoses of vitamin D the first of these factors is of special consequence, while with Ca-deficient diets and with larger excesses of the vitamin, withdrawal from the bone shaft is the main source.

That vitamin D increases absorption of Ca (or P) from the gut (or decreases excretion into the gut) is shown: (1) from the effects of increased Ca intake in hypervitaminosis, (2) from the observation that withdrawal of vitamin excess causes greatly increased faecal excretion of Ca (or P), (3) from recalculations of available data for intake and faecal output, it being shown that each addition of vitamin D to a diet causes an increased net absorption, until ultimately with highly toxic levels a generalised failure complicates the result, and (4) from evidence from rickets and its cure. The mode of action of vitamin D in this respect stands in contrast with that of the parathyroid hormone.

Thus while addition of vitamin D to the diet tends to raise the blood-Ca (or -P, since one influences the other) the actual level so attained is the resultant of several factors: operating in one direction is the increased net absorption and dissolution from certain sites, and, in the opposite, the deposition in other sites, and (when high levels are reached) an increased urinary excretion. With increasing doses of vitamin D the retention by the animal as a whole first rises but ultimately falls, the kidney excretion overtaking the gut absorption.

REFERENCES.

- Adam (1928). *Klin. Woch.* **7**, 1825.
- Aschenheim and Kaumheimer (1911). *Monatsh. Kinderheilk.* **10**, 435.
- Ashford (1930). *Biochem. J.* **24**, 661.
- Bacharach and Jephcott (1929). *J. Biol. Chem.* **82**, 757.
- Baumgartner, King and Page (1929). *Biochem. Z.* **213**, 170.
- Borghi (1929). *Biochim. Terap. Sper.* **16**, 113.
- Brown and Shohl (1930). *J. Biol. Chem.* **86**, 245.
- Cartland, Speer and Heyl (1929). *J. Pharm. Exp. Ther.* **36**, 619.
- Collazo, Varela and Rubino (1928). *Rev. Assoc. Méd. Argent.* **41**, No. 273.
- — — — (1929, 1). *Rev. Sudamericana Endocrinol. Inmunol. Quimioterap.* **12**, 591.
- — — — (1929, 2). *Deutsch. med. Woch.* **55**, 2794.
- — — — (1929, 3). *Wien. Arch. inn. Med.* **137**.
- Comel (1929). *Biochim. Terap. Sper.* **16**, 8.
- Dixon (1929). *Nature*, **124**, 204.
- — and Hoyle (1928). *Brit. Med. J.* ii, 832.
- Duguid, Duggan and Gough (1930). *J. Path. Bact.* **33**, 353.
- Ghirardi (1929). *Biochim. Terap. Sper.* **16**, 241.
- Grayzel and Miller (1928). *J. Biol. Chem.* **76**, 423.
- Greig (1930). *Proc. Eleventh Internat. Vet. Congress.*
- Gyorgy (1929). *Klin. Woch.* **8**, 684.
- Haendel and Malet (1929). *Ann. Fac. Med. Montevideo*, **14**, No. 4.
- Harris (1928). *Science Progress*, **23**, 68.
- — (1930). *Lancet*, i, 236.
- — and Innes (1928-9). *Report of the Med. Res. Council*, 77.
- — and Moore (1928). *Biochem. J.* **22**, 1461.
- — — — (1929, 1). *Biochem. J.* **23**, 261.
- — — — (1929, 2). *Biochem. J.* **23**, 1114.
- — and Stewart (1929). *Biochem. J.* **23**, 206.
- Haury (1930). *J. Biol. Chem.* **89**, 467.
- Havard and Hoyle (1928). *Biochem. J.* **22**, 713.
- Hess, Lewis and Rivkin (1928). *J. Amer. Med. Assoc.* **91**, 783.
- — — — Weinstock and Rivkin (1928). *Proc. Soc. Exp. Biol. Med.* **26**, 199.
- — — — (1929, 1). *Proc. Soc. Exp. Biol. Med.* **27**, 55.
- — — — (1929, 2). *Proc. Soc. Exp. Biol. Med.* **27**, 199.
- — — — (1930). *Proc. Soc. Exp. Biol. Med.* **27**, 298.
- Heubner (1929). *Klin. Woch.* **8**, 407.
- Holtz and Brand (1929). *Z. physiol. Chem.* **181**, 227.
- — and Schreiber (1930). *Z. physiol. Chem.* **191**, 1.
- Hottinger (1929). *Z. Kinderheilk.* **47**, 341.
- Hoyle (1929). *Lancet*, i, 734.
- — (1930). *J. Pharm. Exp. Ther.* **38**, 271.
- — and Buckland (1929). *Biochem. J.* **23**, 558.
- Hughes, Shrivastaya and Sahai (1929). *Indian J. Med. Res.* **17**, 461.
- Jephcott and Bacharach (1926). *Biochem. J.* **20**, 1351.
- — — — (1928). *Biochem. J.* **22**, 60.
- — — — (1930). *Lancet*, ii, 1044.
- Jones, Rapoport and Hodes (1930, 1). *J. Biol. Chem.* **86**, 267.
- — — — (1930, 2). *J. Biol. Chem.* **89**, 647.
- Kreitmaier and Hintzelmann (1928). *Arch. exp. Path. Pharm.* **137**, 203.
- — and Moll (1928). *Munch. med. Woch.* **15**, 637.
- Kroetz (1927). *Klin. Woch.* **6**, 1171.
- Lasch (1928). *Klin. Woch.* **7**, 2148.
- Leané and Clément (1929). *Compt. Rend. Soc. Biol.* **101**, 1023.
- Light, Miller and Frey (1929). *J. Biol. Chem.* **84**, 487.
- Martland and Robison (1924). *Biochem. J.* **18**, 1354.
- Massengale and Nussemeier (1930). *J. Biol. Chem.* **87**, 415.

- Morelle (1930). *J. Physiol.* **70**; *Proc.* xiii.
 Norris and Church (1930). *J. Biol. Chem.* **89**, 444.
 Pfannenstiel (1927). *Klin. Woch.* **6**, 2310.
 Puschler (1929). Quoted in *Lancet* (1930), i, 42.
 Reyer and Walkoff (1928). *Münch. med. Woch.* **75**, 1071.
 Scheunert and Schieblich (1929). *Klin. Woch.* **8**, 983.
 Shipley (1924). *Johns Hopkins Bull.* **35**, 304.
 ——— Kramer and Howland (1926). *Biochem. J.* **20**, 379.
 Shohl, Goldblatt and Brown (1930). *J. Clin. Invest.* **8**, 505.
 Simmonet and Tanret (1929). *Compt. Rend. Soc. Biol.* **100**, 548.
 ——— (1930). *Bull. Soc. Chim. Biol.* **12**, 371.
 Steenbock and Black (1925). *J. Biol. Chem.* **64**, 263.
 Steudel (1929). *Klin. Woch.* **8**, 850.
 Taylor, Branion and Kay (1930). *J. Physiol.* **69**; *Proc.* xxxv.
 Underhill (1928). *Lancet*, ii, 948.
 Vara-Lopez (1930). *Klin. Woch.* **9**, 1072.
 Warkany (1928). *Z. Kinderheilk.* **46**, 1.
 Watchorn (1930, 1). *Biochem. J.* **24**, 631.
 ——— (1930, 2). *Biochem. J.* **24**, 1560.
 Weinmann (1929). *Klin. Woch.* **8**, 841.
 Zucker and Matzner (1923). *Proc. Soc. Exp. Biol. Med.* **21**, 186.

DESCRIPTION OF FIGURES IN PLATES I AND II

PLATE I. Comparative X-ray photographs showing end of long bone (knee joint), (1) normal, (2) in rickets, and (3) in hypervitaminosis.

C = epiphyseal cartilage.

E = epiphyseal end of diaphysis.

1. Normal.
2. Rickets: widening of epiphyseal cartilage and deficient calcification of epiphyseal end of diaphysis.
3. Early hypervitaminosis, 8 days: abnormally narrow epiphyseal cartilage; and broad dense band of excessive calcification at epiphyseal end of diaphysis, extending into marrow cavity.
4. Later stage, 14 days (complicated by cessation of growth and osteogenesis): appearance of a gap between the dense overcalcified area and the epiphyseal cartilage.
5. Same, more advanced, 29 days, with calcification at epiphyseal cartilage C.
6. Cure of early hypervitaminosis: (a) partial loss of excessive calcium, (b) cure complete, bone structure returned to normal.
7. Hypervitaminosis with same level of vitamin D excess but on Steenbock's rachitogenic diet: bone structure almost normal.

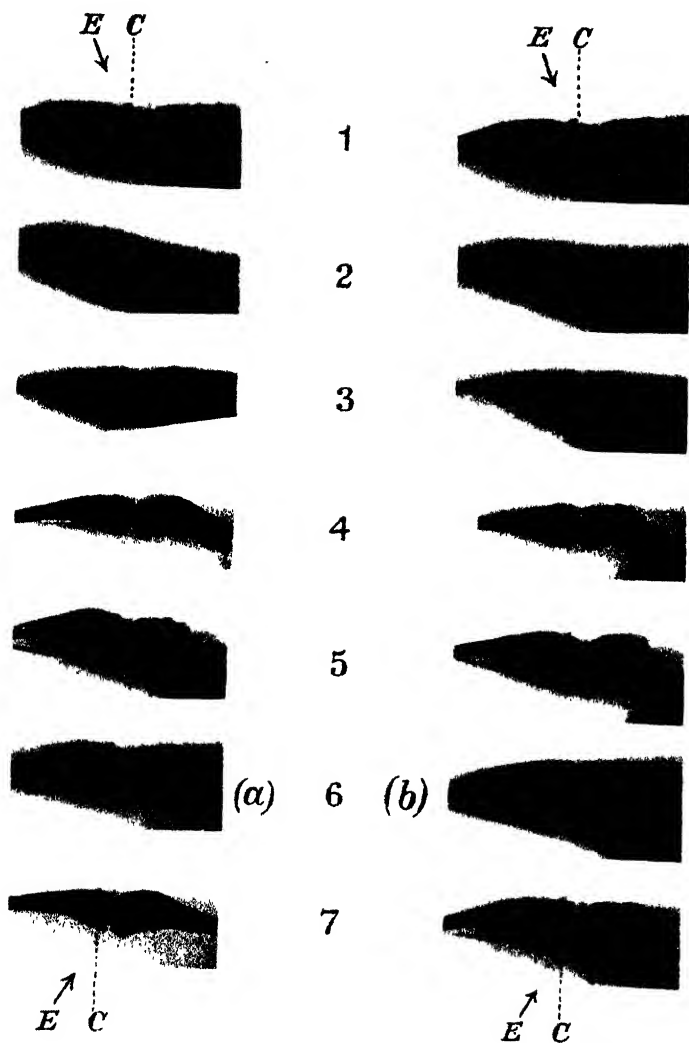
PLATE II. Bones and teeth in hypervitaminosis D (rat).

A. TEETH.

1. Normal control. Note uniform normal dentine (D), cement (C) and jawbone (J). (Transverse section of incisor. Haematoxylin and eosin. $\times 160/3$.)
2. Hypervitaminosis in young rat. Enormous overgrowth of cement (C). Formation of true bone (b) near dentinal junction. Osteoporosis of jawbone (J). Abnormal secondary dentine (D). ($\times 160/3$.)

B. LONG BONE.

1. Normal control. Note features of normal osteogenesis at epiphysis: regular intermediate growth-cartilage (C), showing normal columns of cartilage cells and trabecular formation (T). Normal compact cortex of shaft (S). (Distal end of femur. $\times 50/3$.)
2. Early degree of hypervitaminosis before much resorption had occurred from cortex of shaft (no severe calcification of kidney or aorta was yet apparent at *post mortem*). Decrease in thickness of intermediate growth-cartilage (C). Increased new bone formation: trabeculae covered with thick lamellae of bone (T). (Proximal end of tibia. $\times 50/3$.)
3. Later stage. Thin intermediate growth-cartilage (C). Further great increase in sub-epiphyseal spongiosa (T) extending far into marrow cavity. Thin osteoporosed cortex of shaft (S). Slight resorption (R) of spongiosa nearest growth cartilage. (Distal end of femur. $\times 50/3$.)



Page 890. Lettering on Plate II, A, in figs. 1 and 2:
 "C" should be raised $\frac{1}{8}$ " and $\frac{1}{2}$ " respectively.

Plate II, description of A, fig. 2:

for Enormous overgrowth of cement (C). Formation of true bone (b)

read Enormous overgrowth of cement (C), with formation resembling true bone ("adventitious cement") (b)

Page 492, 10th line from bottom:



21



1



A

B

XLVI. THE ELECTROPHORETIC VELOCITIES OF GELATIN AND OVALBUMIN IN DIFFERENT CONCENTRATIONS OF THEIR MIXTURES AND THE EFFECT OF ULTRA-VIOLET IRRADIATION.

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(Received January 1st, 1931.)

OBSERVATIONS of the motions of proteins in an electric field by many different methods have given results which, as shown in a previous paper [1929], are in substantial agreement. In this communication we have described the conditions under which the motion of a boundary, made visible by means of colloidal gold, may be measured in an improved form of U-tube apparatus [1930].

The investigation has now been extended from 0.1 % solutions of proteins to those mentioned in the title of this paper. In these solutions, differences in potential gradient are enhanced, and inconstancies in the motions of the boundaries are more frequent. The causes of these inconstancies, as they affect hydrophobic sols such as those of metals and sulphides, have been considerably elucidated by the work of Mukherjee [1923] and Krøyt [1925; Krøyt and van der Willigen, 1928].

Mukherjee used a U-tube of the type first described by Burton [1906] in which the sol is run in below the sol-free electrolyte. Many workers have found it difficult to set up a sharp boundary in such a manner. The difficulty has been obviated, for example in our apparatus, by separating the sol from the upper solution of electrolyte by means of taps, which are only opened after complete hydrostatic equilibrium has been established between the two limbs. Also disturbances due to diffusion of the products of electrolysis from the electrodes to the moving boundary are encountered when using the Burton type of tube. These were avoided in our apparatus, and in many others, by setting the electrodes in side-tubes. Any outstanding irregularities in Mukherjee's results are probably due to these causes.

This worker measured potential gradients in various sections of the tube by means of side-tubes sealed in at different levels, and found considerable variations when an arsenious sulphide sol containing free HCl and H₂S was introduced below a KCl solution having the same conductivity. When, however, HCl, having a slightly greater conductivity than that of the sol, was placed above the latter, the potential gradients in the sections immediately

above the original position of the boundary remained nearly constant during an experiment. These potential gradients gradually increased from the top section (HCl) to the lowest section (HCl + sol + H_2S). The average velocities in the lower sections, each corrected for their potential gradients, were in good agreement. They were 3.74 and 3.84 $\mu/\text{sec.}$, v./cm. In the anode limb, where the specifically slower SH^- ion follows the faster Cl^- ion upwards, the boundary remains sharp, while in the cathode limb where the faster ion follows the slower, the boundary becomes diffuse. Yet the calculated velocities in each limb were found to be nearly equal. These results seem to show that, although ionic diffusing layers may mix up the migrating particles, these do not take part in the ionic motion to the extent of being accelerated by the faster ions, *i.e.* the particles do not behave in this respect like slow ions. In agreement with this, it was shown that equivalent concentrations of sulphuric acid gave the same electrophoretic velocities, although there is considerable difference between the mobilities of Cl^- and of $\frac{1}{2}\text{SO}_4^-$. Although this conclusion does not necessarily hold good for a protein micelle, we may assume that it is approximately true for low velocities of the protein.

The general results of Mukherjee may be summarised:

(a) The velocity of movement of a boundary is dependent on the potential gradient of the section of tube in which it is moving.

(b) This potential gradient is determined by the conductivity of the solution in each section, including any conductivity due to the sol particles themselves.

Kruyt and van der Willigen [1928] also investigated sols of As_2S_3 containing up to 22 g. per litre. On these were superimposed intermicellar liquids obtained by ultra-filtration of the sol through collodion. The conductivities of the sols were 2-3 times those of the intermicellar liquids, partly owing to the decomposition of H_2S in the latter, partly owing to the presence of the negative sol particles in the former. The observed velocities in the "down" and "up" columns (cathode and anode limbs) were divided by the potential gradients in the sol and intermicellar liquid respectively, and agreed well. Thus the velocities of a sol containing 17.5 g. As_2S_3 per litre were 4.18-4.25 $\mu/\text{sec.}$ v./cm. at 20°. The potential gradients E_1/l_1 and E_2/l_2 in the sol and solution columns may be calculated from the conductivities, since

$$E_1/l_1 : E_2/l_2 = \kappa_2/\kappa_1.$$

Thus, in one case, $E_1/11.5 : E_2/12.1 = 6.21/16.15$

and E_1 and $E_2 = 75$ v.

Therefore $E_1/l_1 = 1.75$ and $E_2/l_2 = 4.55$ v./cm.

The observed velocity on the upward side was 1.35/900 cm./sec., therefore the velocity under unit potential gradient was

$$\frac{1.35 \times 10^4}{900 \times 4.55} = 3.3 \mu/\text{sec.}, \text{ v./cm.}$$

On the assumption that the velocity is determined solely by the potential gradient, another formula may be deduced which gives the velocity " v " in terms of the observed velocities v_1 (up) and v_2 (down), i.e.

$$v = \frac{v_1 l_1 + v_2 l_2}{E}.$$

We have used this to check some of our results.

It seems probable that the difficulties of determining electrophoretic velocities of hydrophobic colloids have been completely overcome.

As a provisional method of attack and pending a more complete knowledge of the complex protein micelles, it seems justifiable to apply these results to systems containing proteins, bearing in mind that, by virtue of their amphotheric character, these may behave to a greater or less extent as multivalent ions of rather low mobility.

EXPERIMENTAL.

Materials.

Ovalbumin. The whites of eggs, which were not more than 24 hours old, were treated according to the methods developed by Hopkins and Pinkus [1898]. The age of the egg is an important factor, since eggs which are more than 36 hours old do not readily yield a crystalline albumin. The ovalbumin was recrystallised twice and then freed from electrolytes by the dialysis of its solution against distilled water, using a good quality of parchment membrane. A stock solution of 5.14 % concentration was prepared but, owing to the denaturation which could not be prevented, this had to be filtered before use, and the protein content determined by estimating the residue after drying to constant weight at 110°. Toluene was used as a preservative.

Gelatin. Coignet's gold leaf gelatin was purified according to the method of Loeb. A stock solution of 1.85 % concentration was prepared, using toluene-saturated water, and was kept as a gel at 5°. The ash of this material was 0.03 % calculated on the weight of dry gelatin.

Irradiation.

Preliminary experiments on the ultra-violet irradiation of ovalbumin indicated that the molecule underwent a profound change: the possibility that this may play an essential part in the formation of pyrenoids of the Protophyta has already been discussed [Howitt, 1930]. The question of the mechanism of this denaturation is being investigated experimentally by one of us. For the present purpose it will be sufficient to describe the production, by ultra-violet irradiation, of the albumin-gold sol which was to be used for electrophoretic experiments. 300 cc. of a solution containing 0.2 % of pure ovalbumin and 0.02 % of gold (as $\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$) were exposed to a mercury vapour lamp in a quartz flask placed about 5" from the source of light. A slow stream of nitrogen was passed through the solution with the twofold purpose of excluding atmospheric oxygen and agitating the liquid. The irradiation

was continued for 22 hours in periods of 2-3 hours, in order to avoid excessive heating. A splendid gold sol was formed by this irradiation, a gold mirror also being deposited on the walls of the flask. One volume of this solution was taken for the electrophoretic experiments, 0.2*N* acetate buffer was added, and the mixture was diluted to 2 volumes, so that finally it contained 0.1 % of protein, and was 0.02*N* in acetate. It must be pointed out that the product of irradiation of ovalbumin in presence of gold chloride is not necessarily the same as that prepared in absence of gold salt.

Gelatin. A 0.5 % solution was irradiated in a manner similar to that used for ovalbumin, and for 28 hours. A gold sol was then added. The p_H of the solution rose from 4.85 to 6.55. That the protein had altered was shown by the following observations. Precipitation by half-saturated ammonium sulphate was less evident in the irradiated than in the original material. When a 0.1 % solution was allowed to stand in the presence of 0.01 % gold for some weeks, coagulation occurred in certain cases, *viz.*:

p_H	Appearance
2.07	Clear red colour
2.35	"
2.75	Coagulated, blue
3.38	Purplish red colour
3.56	Light red colour, partly coagulated
5.60	Red colour, clear
7.75	Deep red
8.95	"

Thus there appears to be a loss of protective power in two zones of p_H . The coagulation at p_H 2.8 was irreversible with respect to gold, while that which occurred up to p_H 4.5 was reversible.

The gold sol was prepared by two methods.

(a) Bredig's method, by passing an arc between two gold wires in 0.0005*N* HCl [Beans and Eastlack, 1915].

(b) Phosphorus in ether.

No material difference in velocity due to the different preparations was observed.

Electrophoresis.

Our method, as previously described [1929], consisted in the observation of the motion of a boundary between an acetate buffer (0.02*N* in total acetate) above and a protein-gold sol below (0.02*N* in acetic acid or total acetate), which had been brought to the same p_H by the addition of dilute acid or alkali. A potential fall of 222 v. was applied at electrodes of zinc in 5 % zinc chloride and 3 % agar, placed in side-tubes far removed from the boundaries.

The sols contained 0.1 % of protein and 0.01 % of colloidal gold. The concentration of sol must always be kept above that point at which mutual precipitation with the gold occurs. It was shown by Gann [1916] that an acid gold sol prepared according to Donau [1905] is precipitated by gelatin up to concentrations of about 0.0001 % of the latter, and by Freundlich and Loening [1921] that an acid solution containing 0.01 to 0.02 % of gelatin does not

flocculate gold, but that at this concentration the gold still moves electrophoretically towards the positive pole, and therefore behaves as (negative) gold. At higher concentrations, however, 0.05 to 0.10 % gelatin, our results and those of other investigators [Reinders and Bendien, 1928] have shown that the gold migrates to the negative pole, and behaves fully as protein; the velocity changing with change of p_H in the manner which is characteristic of the protein without gold. In alkaline solution it is stated that there is no sensitisation or coagulation of the gold, even when the protein is in very low concentration.

We have also noticed sometimes a coagulation of albumin at or near its isoelectric point in the presence of gold. We consider this to be an entirely different effect from that which takes place in acid solution. The isoelectric effect takes place at high protein concentrations, *i.e.* 1 %. The gold seems to be carried down with the protein and is not changed in colour. Such coagula are easily peptised by a trace of alkali, which is insufficient to make any appreciable alteration in p_H , with the production of an apparently unaltered red sol.

Gelatin lowers the velocity of colloidal gold in all sols and reverses it when $p_H = 4.2$ and when the gelatin has a concentration of more than 0.005 % [Reinders and Bendien, 1928].

Irregularities in the motion of protein boundaries "up" and "down" are partly due to differences in potential gradients. The ratios of the conductivities of sols to those of buffers are higher when the sols contain higher concentrations (0.5-1.0 %) of gelatin and albumin than when they contain lower concentrations (0.1 %). Therefore the ratios of the potential gradients are also higher for the more concentrated protein sols. In the case of albumin, the differences between up and down velocities were greatly reduced by correcting for the potential gradient.

Potential gradients may also be set up by diffusion potentials, which were measured in several instances and found to be 9 or 17 mv. in different acid solutions of p_H 3.6. If the diffusion potentials extend over a length of 1 mm. the gradients will be negligible compared with those of the main applied potential, which amount to several hundred millivolts per mm. The ionic diffusing layers will be spread out or made more diffuse when the current is carried by a quicker ion which is following a slower ion, and from regions of lower to those of higher conductivity. In the acid solutions, the boundary on the anode side moves downward, the faster Na^+ following the slower gel⁻ cation down, and the faster Cl^- following the slower A^- up. The ionic double layer will on the whole become diffuse after the passage of the current. On the cathode side the ionic double layer will be sharper, the Cl^- ions of the sol moving down and leaving the gel⁺ to move up in a region of higher potential gradient, in which also they may be accelerated by the motion of the Na^+ ions. The very high velocities of 3 μ /sec. or more with which the upward boundary sometimes moved may be due to one or other of these causes. On the other

hand, the protein micelles probably carry with them a considerable proportion of the ions which conduct the current, and this will tend to equalise the potential gradients of both boundaries.

These uncertainties seem to be inherent; however we considered that the most important condition is the constancy of p_H throughout the liquid. Differences in p_H not only greatly affect the velocities but also may give much higher diffusion potentials than those observed, which are due to unequal concentrations and mobilities of Na^+ , Cl^- and CH_3COO^- . It is possible, while maintaining p_H constant, to equalise also the conductivities in the two solutions by adding a neutral salt such as KCl to one or both. This device was successfully employed by Hardy [1905] who thus equalised the up and down movements of methylene blue. Kruyt [1925] found however that a gelatin sol had different velocities (a) when sol and solution were at the same p_H , (b) when the conductivities were made the same by addition of neutral salt to the solution. This result was confirmed by special experiments which are described in the series which deals with 1.0 % gelatin.

On account of the possible effect of the addition of neutral salts upon the proteins we have decided not to equalise the conductivities in this way. But, since the correction for potential gradients greatly improves the agreement between the "up" and "down" values, we have determined separately the conductivities of the buffer and sol containing 1 % albumin, and hence have determined the best form in which a mean correction can be made (fourth column of Table I). From the conductivities of the buffer and sol are determined the potential gradients in these parts, as described in the example (below). The means of all the buffer potential gradients are taken, and likewise the mean of all the sols. The mean of these means is used as a divisor of the mean (up and down) velocities at each p_H . The result (fifth column of Table I) agrees in most cases with the values in the fourth column.

Specimen calculations: albumin 1 %. At p_H 4.09.

$$v = +18.5 \text{ (down)} - 31.5 \text{ (up) in mm./hour.}$$

$$= 5.15 \text{ (down)} - 8.75 \text{ (up) in } \mu/\text{sec.}$$

κ_1 (sol) and κ_2 (buffer) are proportional to 0.001136 and 0.000538 respectively.

$$l_1 \text{ (sol)} = 20 \text{ cm.}, l_2 \text{ (buffer)} = 34 \text{ cm.}$$

$$\frac{E_1}{l_1} = \frac{E\kappa_2}{\kappa_2 l_1 + \kappa_1 l_2} = 2.35 = \text{potential gradient in sol,}$$

$$\frac{E_2}{l_2} = \frac{E\kappa_1}{\kappa_2 l_1 + \kappa_1 l_2} = 5.15 = \text{potential gradient in buffer,}$$

$$v_1 = \frac{v \text{ (down)}}{E_1/l_1} = 2.2, \quad v_2 = \frac{v \text{ (up)}}{E_2/l_2} = 1.7.$$

$$\frac{v_1 + v_2}{2} = 2.0 \text{ at } 16.0^\circ \frac{2.0 \times \eta_{18}}{\eta_{18}} = 2.09 \text{ at } 18^\circ.$$

$$\text{Mean uncorrected "v"} = \frac{5.15 + 8.75}{2} = 6.79.$$

$$\text{Mean "v" under unit potential gradient at } 18^\circ = \frac{6.9 \times \eta_{18}}{3.8 \times \eta_{18}} = 1.93.$$

The agreement is not always so good as this. Thus at p_H 2.90, v_1 ($\mu/\text{sec.}$) = 1.67, v_2 = 19.1, E_1/l_1 = 0.83, E_2/l_2 = 6.0. Therefore, at unit potential gradient, v_1 = 1.98, v_2 = 3.2, mean = 2.60 at 17°. In the middle part of the series, from about p_H 4.5 to 8.0, the conductivities of sol and buffer do not differ much from one another, and the "up" and "down" values are in fair agreement, which is usually improved by the correction for potential gradient. There are occasional exceptions such as the velocities at p_H 5.95 which are v_1 = 0.85 and v_2 = 2.0 respectively.

Gelatin 1 % at 28° and p_H 3.63.

v $\mu/\text{sec.}$ + electrode 2.5 (down) -- electrode 7.65 (up).

κ_1 and κ_2 proportional to 4.41×10^{-4} and 2.26×10^{-4} respectively (sol and buffer).

E_1/l_1 = 2.58, E_2/l_2 = 5.0.

$v_1 = \frac{2.5}{2.58} = 0.97$, $v_2 = \frac{7.65}{5.0} = 1.53$.

$v_1 + v_2 = 1.25$ at 28° $\times \frac{\eta_{28}}{\eta_{18}} = 0.99$ at 18°.

Mean uncorrected v = 5.07.

Mean v under unit potential gradient at 18° = $\frac{5.07 \times \eta_{28}}{3.8 \times \eta_{18}} = 1.05$.

The correction for viscosity. A series of measurements of the relative viscosities of albumin, gelatin and their mixtures, at various values of p_H , made in the Ostwald viscosimeter, will be the subject of a further communication. Great differences are found between the viscosities of 0.5 % gelatin and albumin on the one hand and 0.5 % and 0.25 % gelatin on the other. Since the velocities of the 1.0 % gelatin and albumin and of the 1.0 and 0.1 % gelatin respectively agree in the main, and a correction on the supposition that the values of " v " are inversely proportional to these viscosity figures, *i.e.* under low rates of shear, would give the curves quite a different character, in the equation

$$v = \frac{1}{4\pi} \frac{HD\xi}{\eta},$$

it has been assumed that the viscosity η is really that under high shearing stresses, *i.e.* from 400 mm. Hg upwards [Abramson, 1928]. It has also been observed by Freundlich, Abramson and others that the electrophoretic velocities of red blood corpuscles are almost unaffected by great variations in the viscosities of media containing serum, gelatin *etc.*, even by the very high values which are found after gelation has occurred.

Since electrophoresis is due to movements of ionic double layers, it is generally considered that the viscosity is that which affects and varies inversely as the conductivity. It has been found that the addition of a neutral substance, such as starch, in amounts sufficient to produce a very high viscosity has only a slight effect on conductivity [Hardy, 1905].

Since also the temperature coefficient of viscosity is the inverse of and nearly the same as that of conductivity, the correction, *e.g.* to 18°, may be introduced

either as κ_{18}/κ_t or η_t/η_{18} . It is known that the electrophoretic velocities of silver sols, determined at different temperatures, become constant when corrected to a constant temperature [Burton, Physical properties of colloidal solutions]. Abramson [1929] assumes a temperature coefficient of electrophoretic velocity of 2 % per 1°.

We have thought it best to correct the results simply for the alteration in viscosity of water. In our former paper, the velocities were referred to those at 15° as standard. The present series have been referred to 18°, so that the former values are multiplied throughout by the factor η_{15}/η_{18} for comparison.

Table I. *Albumin 1 %.*

pH	Temperature	v in μ /sec. Mean	$r/3.8$ Mean velocity divided by mean potential gradient and corrected to viscosity at 18°	$\frac{r_1 l_1 / E_1 + r_2 l_2 / E_2}{2}$ Mean of "up" and "down" velocities each divided by its potential gradient and corrected to viscosity at 18°
2.50	18.0°	6.70	+1.76	+2.25
2.90	16.5	10.38	2.83	2.70
3.56	18.5	7.80	2.05	2.56
4.09	16.0	6.90	1.90	2.10
4.44	18.5	4.45	1.16	1.15
5.42	16.0	1.65	-0.45	-0.41
5.95	19.0	4.95	1.26	1.33*
6.48	18.5	4.50	1.17	1.14
6.58	17.0	4.03	1.08	1.02
7.03	19.5	6.40	1.62	1.59
8.24	20.0	6.30	1.57	1.53
9.38	19.5	7.55	1.91	1.92

* Not plotted in Fig. 1.

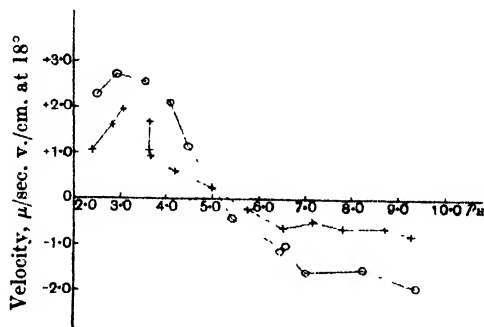


Fig. 1.

—○— 1.0 % ovalbumin. -+- 1.0 % gelatin.

Comparison of 1 % gelatin and albumin.

Our previous results, in 0.1 solution, which are included in Table III, seem to show a close agreement between the electrophoresis of the two proteins. A comparison is more difficult in the case of the 1 % solutions on account of the irregularities on the acid side, and the necessity of carrying

Table II. *Gelatin 1 % at 28°.*

pH	v in mm./hr. Mean at 28°	" v " in μ /sec. v./cm. at 18°
2.37	19.0	+1.09
2.80	27.5	1.58
3.06	31.7	1.94*
3.63	18.2	1.04
3.63	27.0	1.67†
4.19	9.5	+0.55
4.97	4.0	+0.23
5.77	3.0	-0.19‡
6.48	11.0	0.63
7.15	9.0	0.51
7.83	11.0	0.63
8.70	11.0	0.63
9.30	14.0	0.80

* 1 % gelatin at 25.0.

† 0.8 % gelatin at 25.0. The conductivities of sol and buffer were made nearly the same by the addition of KCl to the buffer.

‡ 1 % at 25°. Both buffer and sol were 0.1N with respect to KCl, which nearly equalised the conductivities.

Table III. *Gelatin, ovalbumin and their mixtures, 0.1 % in total protein.*

All velocities expressed as in previous tables.

Albumin 0.1 %				Gelatin 0.1 %				Albumin 0.05 %, gelatin 0.05 %			
pH	t	v in mm. hr. at t°	v in μ /sec. at 18°	pH	t	v in mm. hr. at t°	v in μ /sec. at 18°	pH	t	v in mm. hr. at t°	v in μ /sec. at 18°
2.35	15°	5	+0.36 ₅	2.75	15.0	+40.0	+3.15	2.77	16.5	±0.0	0.0
3.56	"	22	1.61	3.70	15.0	16.2	1.25	3.17	16.5	0.0	0.0
3.95	"	16	1.17	4.60	15.0	5.0	+0.39	—	—	—	—
4.45	"	8	+0.58	4.95	16.0	0.0	±0.00	3.90	15.0	+4.0	+0.29
5.56	"	12	-0.88	5.45	15.0	4.5	-0.35	4.22	19.0	3.5	0.25 ₅
5.95	"	13	0.95	5.93	17.0	8.2	0.63	4.72	19.0	2.5	0.18
6.55	"	16.2	1.18	6.60	20.0	8.0	0.55	4.66	16.5	2.0	0.15
7.00	"	26	1.90	7.15	15.0	6.7	0.52	4.94	16.5	1.2 ₅	0.10
7.65	"	24.2	1.86	8.02	21.0	10.0	0.67	5.55	15.0	2.8	0.39
				9.27	20.0	11.0	0.86	6.38	15.0	16.0	0.78
								6.98	15.0	8.0	0.63
								7.70	15.0	9.0	0.78
								8.66	16.0	10.5	0.81
								9.55	16.0	10.5	0.81

The velocities in mm./hour are the means of "up" and "down" values, exclusive of those which had diffused. The velocities in μ /sec. are calculated for unit potential gradient.Table IV. *Albumin 0.1 % and gelatin 0.1 % after irradiation.*

Albumin				Gelatin			
pH	t	v in mm./hr. at t°	v in μ /sec. v./cm. at 18°	pH	t	v in mm./hr. at t°	v in μ /sec. v./cm. at 18°
2.63	15.0°	15.2	+1.2	2.07	17.0°	38.0	+2.84
3.19	16.0	21.0	1.6	2.35	18.0	37.5	2.74
3.90	17.0	21.2	1.6	2.84	20.0	32.7 ₅	2.27
4.55	17.5	7.8	+0.6	3.38	19.5	19.5	1.37
5.72	15.0	12.5	-1.0	3.56	20.5	18.2 ₅	+1.24
6.30	18.0	15.0	1.1	5.60	20.5	22.2 ₅	-1.52
7.54	19.0	22.5	1.6	7.75	20.0	22.2 ₅	1.54
9.30	19.5	27.7	2.0	8.95	19.5	21.0	1.47
				9.34	19.0	27.2 ₅	1.93

out the gelatin experiments at a higher temperature, 25° or 28°. On the whole it appears that the velocities at each p_H are lower in the case of gelatin. This may be connected with the much higher viscosities of gelatin solutions which we hope to investigate further by another method.

In the case of both proteins, velocities seem to reach maxima at p_H 3.0–3.5, then to fall off, in the case of gelatin, very rapidly, so that they even become zero at still lower p_H values. In this connection it may be noted that the combination curve of 1 % gelatin (p_H plotted against x cc. of HCl added) seems to show that the maximum amount is combined at about p_H 2.5, but the acid blank correction is large here so that the amount actually combined is rather uncertain [Atkin and Douglas, 1924].

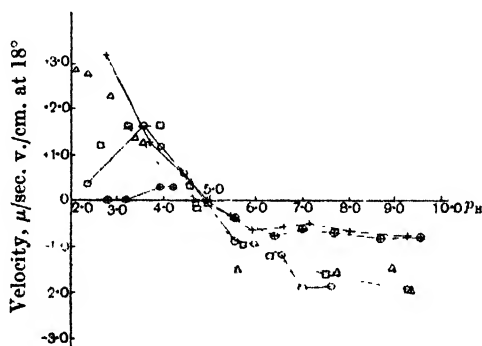


Fig. 2.

- | | |
|-----------------------|-------------------------------|
| ○—○ 0.1 % ovalbumin. | □— 0.1 % irradiat. ovalbumin. |
| +—+ 0.1 % gelatin. | △— 0.1 % „ gelatin. |
| ⊙—⊙ 0.05 % ovalbumin. | |
| ⊕—⊕ 0.05 % gelatin. | |

The isoelectric point of gelatin determined graphically seems to lie above p_H 5.0. On account however of the low values of $\Delta v/\Delta p_H$ on either side of this point, a small alteration in the somewhat irregular velocities of positively charged gelatin would make a large difference in the point at which the line cuts the axis of zero velocity.

On the alkaline side, the velocities “up” and “down” are in good agreement, and as previously found in the case of 0.1 % solutions, become nearly constant at about 0.5–0.7 μ /sec. between p_H 6.0 and 6.5. Referring again to the combination curve of gelatin we find, after p_H 6.0, a rapid increase of p_H with added alkali, showing that the formation of alkali gelatin, or, according to the newer view, the displacement of the weaker set of basic groups from combination with OH, has reached a maximum. Therefore, no additional charge on the micelle is to be expected until higher values of p_H are reached. In the curve of 1 % gelatin [Atkin and Douglas, 1924], buffer action increases again at about p_H 9.5. This is also about the point where in our results an increase in negative velocity reappears.

In the case of 0.1 % solutions of albumin, the negative velocities appear

to increase with increasing p_H at about the same rate as that at which the positive velocities increase with diminishing p_H . The velocities on the alkaline side were more irregular in the case of the 1.0 % solutions. They increased with p_H up to $1.0\mu/\text{sec.}$ or more, at p_H 6, and at higher values of p_H , continued to increase, although more slowly. They remained considerably above the velocities of 1 % gelatin at the same p_H .

Mixtures of 0.05 % gelatin and 0.05 % albumin.

The mixtures contained as usual 0.01 % of gold sol and were adjusted by addition of acid and alkali to the p_H of the 0.02*N* acetate buffers. The results in Table III are compared with those of the 0.1 % proteins measured separately. It might be expected that the motion would be intermediate between those of the separate proteins, or that it would be the same as that which has the greater velocity at each p_H , since each protein should carry with it sufficient gold to make the margin visible. Neither expectation was realised.

On the alkaline side, the protein behaves almost exactly like gelatin, which has the lower velocity. The tendency of the albumin to combine with increasing OH^- concentrations, giving an increasingly negative micelle, is somehow masked by the gelatin, which hardly increases its negative charge in this range of p_H .

On the acid side, the mixture does not behave like either protein. The velocities become somewhat irregular—a negative value having been registered at the isoelectric point 4.7, which according to our results in 0.1 % solution should have given not a zero, but a positive velocity. At p_H 4.0 a slight positive velocity was noted, but at lower p_H values the velocities fell to zero. It appears as if the mixture forms a more stable amphoteric ion, which is not appreciably opened up by the addition of acids. The viscosities of the mixtures, which will be the subject of a further communication, are to some extent intermediate but nearer to those of albumin.

The irradiation does not appear to have made any material alteration in the electrophoretic velocities of either protein. On account of the coagulation, velocities cannot be observed so close to the isoelectric point of the irradiated gelatin. A small portion has undoubtedly been rendered extremely sensitive to oxidation by the gold chloride, and has been destroyed, but the remainder seems to retain the same positive or negative charges.

SUMMARY.

1. The mean velocities of albumin are higher than those of gelatin both in acid and in alkaline solutions.
2. Neither the curve of gelatin nor that of albumin is symmetrical on the acid and alkaline side of the isoelectric point.
3. On the acid side the velocities increase rapidly with diminishing p_H to a maximum, and then fall off.

4. On the alkaline side the velocities increase up to p_H 6-7, then fall off and reach a constant or nearly constant value, which is lower and more nearly constant in the case of gelatin.

5. Mixtures of gelatin and albumin behave anomalously.

REFERENCES.

- Abramson (1928). *J. Gen. Physiol.* **11**, 743.
— (1929). *J. Gen. Physiol.* **12**, 711.
Atkin and Douglas (1924). *J. Soc. Leather Trades Chem. (Collegium)*, 584.
Beans and Eastlack (1915). *J. Amer. Chem. Soc.* **37**, 2667.
Burton (1906). *Phil. Mag.* (6), **11**, 440.
— Physical properties of colloidal solutions (London).
Donau (1905). *Monatsh. Chem.* **26**, 525.
Freundlich and Loening (1921). *Festschr. Kaiser Wilh. Ges.*, Berlin, p. 82.
Gann (1916). *Kolloidchem. Beih.* **8**, 251.
Hardy (1905). *J. Physiol.* **33**, 251.
Hopkins and Pinkus (1898). *J. Physiol.* **23**, 130.
Howitt (1930). *Nature*, **125**, 412.
Kruyt (1925). *Kolloid Z.* **37**, 358.
— and van der Willigen (1928). *Kolloid Z.* **44**, 22.
Mukherjee (1923). *Proc. Roy. Soc. Lond. A* **103**, 102.
Prideaux and Howitt (1929). *Proc. Roy. Soc. Lond. A* **126**, 126.
— — (1930). *J. Sci. Instr.* **7**, 89.
Reinders and Bendien (1928). *Rec. Trav. Chim.* **47**, 977.

XLVII. ON THE NATURE OF THE CELL WALL CONSTITUENTS OF *LAMINARIA* SPP. MANNURONIC ACID.

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THE nature of the cell wall constituents of the red and brown algae has been the subject of study in this laboratory for some time past [Haas and Hill, 1921; Haas, 1921; Russell-Wells, 1922; Haas and Russell-Wells, 1923, 1929]. The present investigation, which is concerned primarily with *Laminaria* spp., was undertaken with the object of throwing some light on the mode of occurrence within the plant of some of the substances which have from time to time been described as being obtained from this genus. The search for the source of the methylpentose, fucose, in particular, has involved the study of the products of hydrolysis of two cell wall constituents known respectively as fucoidin and alginic acid and the results obtained are described herein.

Fucoidin.

The term fucoidin was first applied by Kylin [1913] to the highly mucilaginous material which exudes from freshly collected samples of *Laminaria* spp. and from *Fucus serratus*, *F. vesiculosus* and *Ascophyllum nodosum*. He found it to be a calcium salt and observed that when extracted from the weed with hot water the material was much less viscous; this fact he attributed to the conversion of the calcium salt into the alkali metal salt, though no evidence was offered for his assertion; he stated further that fucoidin contains pentoses and methylpentoses and established the presence of fucose among the products of hydrolysis of this substance.

Some years ago Haas and Russell-Wells [1923] obtained evidence of the existence of an ethereal sulphate grouping in *Laminaria*, and it has now been possible to show that this grouping is actually contained in fucoidin. The method employed for the isolation and purification of this substance was as follows.

The freshly gathered weed was soaked in distilled water whereby a very translucent and highly viscous extract was produced from which, by addition of an equal volume of absolute alcohol, a gelatinous precipitate was obtained. This substance when placed in water again swelled up and went into solution, but gradually lost its gelatinous character on keeping; it was purified by solution in water and re-precipitation by means of alcohol; but with the

gradual removal of salts, precipitation by alcohol became increasingly difficult and this method of purification was abandoned in favour of dialysis. When all dialysable salts had been removed the dialysed solution was poured into alcohol and the precipitated material was preserved in alcohol, since drying caused shrinkage to a tough horny material, which though still soluble in water had a greatly reduced swelling power.

On incineration the material yielded 30.93 % of ash, consisting for the most part of calcium sulphate. The calcium was shown to occur in the original material in the ionised condition, being precipitable by ammonium oxalate. That the substance was an ethereal sulphate was shown by the fact that the original material dissolved in water gave no reactions for the sulphate ion but that after hydrolysis it yielded precipitable sulphate in approximately double the quantity of that in the ash.

$$\begin{array}{rcl} \text{Sulphate in hydrolysed solution} & = & 30.33 \% , \\ \text{ash} & = & 15.10 \% . \end{array}$$

The presence of methylpentose, described by Kylin, was confirmed both by the colour reaction of Rosenthaler and by the isolation of an osazone crystallising in yellow needles, M.P. 170–173°, from the products of hydrolysis of the material with 3 % sulphuric acid in a boiling water-bath for 11 hours. The preparation from *Fucus* spp. of fucose was first described by Günther and Tollens [1890] and later by Votoček [1915] and by Clark [1922] who hydrolysed the entire weeds, after a preliminary soaking in dilute sulphuric acid. In view however of the evidence here presented for the occurrence of fucose in the water-soluble fucoidin of *Laminaria*, it appears that the preliminary soaking in dilute acid may remove some of the potential source of this sugar¹. Heated with 12 % hydrochloric acid fucoidin produced furfuraldehyde which yielded a black phloroglucide soluble in alcohol; carbon dioxide was likewise given off, corresponding to about 7.3 % of a uronic acid complex, but, owing to the limited amount of material available, no attempt was made to ascertain its nature.

Algin and alginic acid.

The term algin was first applied by Stanford [1883, 1884, 1886] to a mucilaginous material occurring in *Laminaria* fronds; on extracting the weed with cold 2 % sodium carbonate and acidifying the resulting solution he obtained a precipitate which he described as alginic acid; of this acid he prepared a number of salts including an insoluble calcium salt containing 7.84 % of calcium.

Kylin [1915] applied the term algin to a supposed insoluble calcium salt which he assumed to occur in the cell wall; he stated that it was insoluble

¹ The completely exhausted residue from the extraction of 200 g. of *Laminaria* fronds with 2 % sodium carbonate, weighing 80 g., when hydrolysed under the conditions described by Clark [1922] for *Fucus vesiculosus*, yielded a very small quantity of an osazone which could not be definitely identified with that of fucose. From this fact it would appear that in the case of *Laminaria* at all events, the chief source of fucose would be the fucoidin.

or very sparingly soluble in water and suggested that hot water extracted from the weed an alkali metal salt of alginic acid produced from the calcium salt, but he did not support this by any experimental evidence. His explanation, moreover, of the action of sodium carbonate in extracting alginic acid was that it underwent double decomposition with the insoluble calcium salt yielding soluble sodium alginate.

In these circumstances it seemed worth while to examine the nature of the material extracted by hot water and to ascertain whether there were any limit to the extent to which the alginic acid contained in the cell wall could be extracted by this method. To this end 180 g. of *Laminaria* fronds were cut up, tied in a calico bag and extracted with boiling water. In view of the fact that water would also extract fucoidin, the first extracts were rejected until the absence of fucoidin could be established by hydrolysing with hydrochloric acid and showing that no sulphate was formed. The extraction was then continued until the extracts contained only negligible amounts of material; they were then evaporated to small bulk and precipitated with alcohol. The precipitated material was dissolved in a smaller volume of water and dialysed; after dialysis was complete, the material could no longer be precipitated by the addition of alcohol and was therefore recovered from solution by evaporation on a water-bath, when it weighed 2 g. Thus dried it would no longer dissolve in water. Analysis showed that it contained 3.46 % Ca and 0.45 % Mg, together with undetermined quantities of alkali metals. From these facts it was taken to be a water-soluble calcium, magnesium, sodium and potassium salt of alginic acid, since it gave, on acidification, a gelatinous precipitate in every way resembling alginic acid. That the extraction with hot water had not removed all the alginic acid from the cell wall was shown by the fact that the residue remaining, when treated with cold 2 % sodium carbonate, at once yielded an extract from which a copious precipitate of alginic acid could be obtained on acidification.

Direct evidence as to the correctness, or otherwise, of Kylin's assumption that alginic acid occurs in the cell wall in the form of an insoluble calcium salt is not easy to furnish, although the readiness with which cold dilute sodium carbonate extracts this acid from the weed is rather against this assumption. The following facts however provide indirect evidence which casts doubt on the occurrence of the calcium salt. From the loss in weight by 200 g. of the weed on exhaustive extraction with 2 % sodium carbonate it was estimated that *Laminaria* contains about 60 % of its weight of alginic acid. Such a proportion of this acid, if occurring as assumed by Kylin, in combination with calcium, would require a calcium content of the weed of 5.1 %, calculated on the basis of the figure given by Stanford for the percentage of calcium in the calcium salt. The calcium content of the whole weed was however found by analysis to be only 1.99 %.

From the above facts it is concluded that the cell wall of *Laminaria* contains alginic acid in two forms, one a soluble calcium, magnesium, alkali metal

salt, present only to the extent of about 1 %, and the other in the free state which appears to be the material extracted by cold dilute sodium carbonate; there does not thus appear to be any need to assume, as Kylin did, that the bulk of the alginic acid occurs in the form of an insoluble calcium salt.

Hydrolysis of alginic acid.

The first attempt to investigate systematically the hydrolysis of alginic acid was undertaken by Hoagland and Lieb [1915]. These authors obtaining their alginic acid from *Macrocystis pyrifera* subjected it to hydrolysis with hydrochloric acid and claimed to have obtained evidence that this substance yielded pentoses which were somewhat doubtfully identified by means of phenylhydrazine as xylose and arabinose. Some years later Schmidt and Vocke [1926] in an examination of alginic acid prepared from *Fucus serratus* described the hydrolysis of this material by successive treatments with anhydrous formic acid, 80 % sulphuric acid, and finally boiling 4.5 % sulphuric acid; they claimed to have established that alginic acid is a polymerised glycuronic acid by preparing a cinchonine salt having m.p. 204°, which is the m.p. given by Neuberg [1900] for the cinchonine salt of glycuronic acid prepared by the hydrolysis of euxanthone.

In view of these conflicting results we decided to reinvestigate the hydrolysis of alginic acid with a view to ascertaining whether it could be regarded as a potential source of pentoses or even of fucose.

For our experiments we chose sulphuric acid in preference to the hydrochloric acid employed by Hoagland and Lieb. The alginic acid¹ was heated under reflux with 8 times its weight of 2 % sulphuric acid in a boiling water-bath for 3 hours. At the end of this time the acid solution was filtered from the insoluble residue A (see below). On removing the sulphuric acid with barium carbonate it was found that the filtrate contained considerable quantities of barium in solution indicating that an acid forming a soluble barium salt had been produced.

In order to ascertain whether any hexose or pentose sugars had been produced as well, as had been suggested by Hoagland and Lieb's experiments on alginic acid from *Macrocystis pyrifera*, the barium salt in solution was precipitated by addition of alcohol. The aqueous alcoholic filtrate was filtered and evaporated to dryness under reduced pressure; the resulting light brown residue containing barium proved to be some of the barium salt which had escaped precipitation. In order however to ascertain whether it contained any sugars as well, it was repeatedly extracted with 80 % alcohol. Hardly any material was dissolved, but the combined extracts were evaporated and were found still to contain barium. Addition of phenylhydrazine acetate to the cold solution gave an immediate precipitate similar to the one described

¹ For much of the alginic acid used in these experiments we have to thank Messrs Nobel and Co., Explosives Ltd., Ardeer, Scotland.

on p. 409 and this proved to be insoluble in all organic solvents, showing that it contained no sugar hydrazones. The absence of sugars was thus established, and consequently alginic acid has to be excluded as a possible source of fucose.

Our results therefore failed to corroborate the observations of Hoagland and Lieb that alginic acid gave rise to pentoses on hydrolysis. In order to ascertain whether alginic acid from *Macrocystis pyrifera* was in any way different from that of *Laminaria* we procured some of the former weed from California through the good offices of the Californian Academy of Sciences, and on subjecting it to the same conditions of hydrolysis with sulphuric acid as described above we obtained substantially the same result, namely a solution containing a soluble barium salt. On treating this solution with phenylhydrazine as described by these authors we obtained in the cold a red-brown precipitate which contained barium but were unable to establish the presence of sugars.

In view of the statement by Schmidt and Vocke referred to above it seemed likely that the barium salt produced in our experiments might be that of glycuronic acid. On preparing the cinchonine salt by addition of the calculated amount of cinchonine sulphate to the barium salt we obtained a substance whose melting point, 195–197° (on one occasion 203°), seemed sufficiently close to that quoted by Schmidt and Vocke to suggest that it actually was cinchonine glycuronate; the specific rotation however, was $[\alpha]_D = 112.8^\circ$ as compared with $[\alpha]_D = 138.6^\circ$ as given by Neuberg. On preparing the quinine salt¹ an even greater discrepancy was found; the constants given by Neuberg for quinine glycuronate are m.p. 180° and $[\alpha]_D = -80.1^\circ$ and the analysis indicated the formula $C_{28}H_{34}O_9N_2$. Our salt on the other hand had m.p. 162–163° and $[\alpha]_D = -175.3^\circ$, while the analysis showed that the substance contained two molecules of water of crystallisation, as may be seen from the following figures²:

	C %	H %
Found	56.01	6.92
Calc. for $C_{28}H_{34}O_9N_2 \cdot 2H_2O$	56.32	6.86

The residue A (p. 406) remaining after the hydrolysis of alginic acid for 3 hours with 2 % sulphuric acid was then subjected to further hydrolysis, with a view to obtaining an increased yield, by heating with 5 % sulphuric acid for 8 hours. After removal of the sulphuric acid a new cinchonine salt was obtained having m.p. 161° and $[\alpha]_D = 154^\circ$; this substance gave the following results on analysis:

	C %	H %	N %
Found	59.21	6.87	5.4
Calc. for $C_{31}H_{38}O_8N_2 \cdot H_2O$	59.29	6.71	5.33

This salt therefore differed from that described by Schmidt and Vocke both in m.p. and in composition, since it contained a molecule of water of

¹ See Addendum.

² All the micro-analyses were carried out by Dr Ing. A. Schoeller of Berlin.

crystallisation. It was produced in much smaller yield than the cinchonine salt m.p. 195–197°. No explanation is at present offered for the existence of these two salts, more particularly since only one quinine salt has, so far, been obtained, but in any case neither of them agreed in physical constants with cinchonine glycuronate.

There seemed therefore no doubt that the acid produced by the hydrolysis of alginic acid from *Laminaria* was not glycuronic acid; as moreover the constants obtained did not agree for the cinchonine salt of galacturonic acid and furthermore alginic acid on oxidation yielded no trace of mucic acid, it seemed natural to conclude that the acid in question might be the hitherto unknown mannuronic acid, especially in view of the plentiful occurrence of mannitol in *Laminaria* and allied fucoids.

To settle this question experiments were undertaken¹ to ascertain the nature of the dicarboxylic acid produced on oxidation of the aldehydo-acid in question. To this end both alginic acid and its product of hydrolysis were oxidised and in each case mannosaccharic acid was identified as the diamide, thus proving conclusively that the aldehydo-acid was not glycuronic acid but the hitherto undescribed mannuronic acid.

Oxidation to mannosaccharic acid.

(a) *Alginic acid.* Earlier experiments on the oxidation of alginic acid by heating on a water-bath with nitric acid in search of the possible formation of mucic acid failed to produce anything but oxalic acid. By carefully regulating the oxidation however a different result was obtained.

5 g. of dried and finely ground alginic acid were covered with 7.5 g. of nitric acid (sp. gr. 1.2); the acid was at once absorbed leaving the alginic acid as a coarsely granulated mass; this was heated under a reflux condenser for 24 hours at 50°; about 50 cc. of water were now added and after thorough mixing the insoluble residue was filtered off on a Büchner funnel. The filtrate was then rapidly evaporated over a water-bath to about half its volume and then to dryness in a desiccator over sodium hydroxide, when a light lemon-yellow syrup resulted. On stirring this with concentrated ammonia it turned brown and deposited in the course of a few minutes a mass of crystals which on recrystallising from water separated in well formed rhombs melting at 188–189°.

Analysis gave the following results:

	C %	H %	N %
Found	34.39	5.84	13.21
Calc. for $C_6H_{12}O_6N_2$	34.61	5.77	13.46

¹ Experiments to this end were already in progress when we accidentally came across a paper by Cretcher and Nelson [1929], whose publication we had overlooked, in which these authors had already come to the same conclusion and had actually established the production of mannosaccharic acid on oxidation. Notwithstanding this we completed our investigation and now publish our results because they were arrived at independently and by slightly different methods.

(b) *Mannuronic acid*. 5.4 g. of barium mannuronate dissolved in 20 cc. of water were freed from barium by the addition of the requisite amount of dilute sulphuric acid and, after filtering, the solution was treated with 16 g. of bromine. The mixture was thoroughly shaken at intervals during $4\frac{1}{2}$ days, after which the excess of bromine was removed by a rapid current of air until the liquid was of a light straw colour. The liquid was then diluted and treated with freshly precipitated silver oxide until neutral and then with hydrogen sulphide to precipitate any dissolved silver as sulphide. The filtrate evaporated to dryness in a desiccator left a colourless viscous residue which on addition of concentrated ammonia deposited rhombic prisms; these after crystallising twice from water melted at 188–189°.

Mannuronic acid.

The oxidation experiments described above showed undoubtedly that alginic acid gave rise on hydrolysis to mannuronic acid, since oxidation of the product led to mannosaccharic acid, identified by comparison of the M.P. of its diamide with that of an authentic specimen and the determination of a mixed melting point.

A sample of the mannuronic acid prepared from its barium salt left a light amber-coloured resin which did not crystallise. An attempt further to characterise the barium salt by treatment of the cold aqueous solution with phenylhydrazine acetate resulted in the production of an immediate red-brown precipitate containing barium. This material on extraction in a Soxhlet with chloroform lost its red colour and became orange-coloured. This substance was insoluble in organic solvents but dissolved sparingly in boiling water separating again rapidly on cooling in spherical aggregates. In spite of repeated attempts, no concordant analyses could be obtained indicating a homogeneous substance. This observation agrees with the experience recorded by Neuberg and Neimann [1905] of difficulty in obtaining a uniform product by the action of phenylhydrazine upon glycuronic acid.

Attempts to prepare a *p*-bromophenylhydrazone resulted in the production of brown resin. Similarly attempts to prepare a crystalline dinitrophenylhydrazone were unsuccessful.

CONCLUSION.

An attempt has been made to elucidate the nature of the cell wall constituents of the Laminariaceae known as fucoidin and alginic acid. The former has been shown to be the calcium salt of a sulphuric acid ester of a non-reducing polymerised uronic acid complex combined with a methylpentosan, since it yields on hydrolysis sulphate ions and the methylpentose fucose; the nature of the uronic acid has not been established.

Alginic acid has been shown to be a polymerised form of the hitherto undescribed mannuronic acid, and to contain no pentosan or methylpentosan groupings. These facts emphasise the distinctive metabolism of the brown

algae, which appears to be founded upon a mannose, rather than a glucose basis—as is shown by the known occurrence of mannitol and mannitan [Haas and Hill, 1929], and the apparent absence of glucose in any appreciable amounts.

SUMMARY.

1. Evidence is furnished that the substance described by Kylin as fucoidin is an ethereal sulphate. The substance contains also some uronic acid complex the nature of which has not been determined.

2. Alginic acid occurs mainly as such in the free state in the cell wall and to a limited extent only in the form of a water-soluble calcium magnesium alkali metal salt.

3. Alginic acid from *Laminaria* yields on hydrolysis mannuronic acid whose quinine salt is here described for the first time (see Addendum).

No evidence could be obtained of the formation of sugars by the hydrolysis of alginic acid and this substance cannot therefore be regarded as a source of any of the sugars which have been obtained by the hydrolysis of *Laminaria* weed as described by Muther and Tollens [1904], Manske [1930] and others.

4. Alginic acid on oxidation with nitric acid yields mannosaccharic acid.

5. Fucose obtained by the hydrolysis of the complete weed of *Laminaria* is derivable in part from fucoidin and in part possibly from the cell wall residue remaining after exhaustive extraction with sodium carbonate, but none is produced from alginic acid.

ADDENDUM (March 27th, 1931).

Since the above was written it was found that when the solution obtained by heating alginic acid with 2 % sulphuric acid was further heated for 8 hours with 5 % sulphuric acid the resulting liquid, on treatment with cinchonine sulphate, yielded the low-melting salt, m.p. 161° and $[\alpha]_D = 154^\circ$. This shows definitely that the nature of the cinchonine salt obtained depends upon the strength of acid and the time employed in the hydrolysis, and that the cinchonine salt of low melting point is not obtained solely from the further hydrolysis of the residue remaining after initial treatment with 2 % sulphuric acid (as implied on p. 407), but may actually be obtained by further hydrolysis of the solution which previously yielded the salt of high melting point.

Attempts to prepare two different quinine salts from the two sources which yielded the two distinct cinchonine salts gave the following results: hydrolysis with 2 % acid yielded a quinine salt of m.p. 168° and $[\alpha]_D = -173.5^\circ$, while hydrolysis with 5 % acid yielded a quinine salt of m.p. 162–163° and $[\alpha]_D = -175.3$. The difference between the physical constants in these two cases is hardly sufficient to justify the conclusion that the two salts are really distinct substances, but on the other hand the existence of two cinchonine salts requires the existence of two quinine salts.

REFERENCES.

- Clark (1922). *J. Biol. Chem.* **54**, 65.
Cretcher and Nelson (1929). *J. Amer. Chem. Soc.* **51**, 1914.
Günther and Tollens (1890). *Ber. deutsch. chem. Ges.* **23**, 1753, 2585.
Haas (1921). *Biochem. J.* **15**, 469.
—— and Hill (1921). *Ann. Applied Bot.* **7**, 352.
—— ——— (1929). *Biochem. J.* **23**, 1000, 1005.
—— and Russell-Wells (1923). *Biochem. J.* **17**, 696.
—— ——— (1929). *Biochem. J.* **23**, 425.
Hoagland and Lieb (1915). *J. Biol. Chem.* **23**, 287.
Kylin (1913). *Z. physiol. Chem.* **83**, 190.
—— (1915). *Z. physiol. Chem.* **94**, 337.
Manske (1930). *J. Biol. Chem.* **86**, 571.
Müther and Tollens (1904). *Ber. deutsch. chem. Ges.* **37**, 298.
Neuberg (1900). *Ber. deutsch. chem. Ges.* **33**, 3321.
—— and Neimann (1905). *Z. physiol. Chem.* **44**, 97.
Russell-Wells (1922). *Biochem. J.* **16**, 578.
Schmidt and Vocke (1926). *Ber. deutsch. chem. Ges.* **59**, 1585.
Stanford (1883). *J. Chem. Soc.* **44**, 943.
—— (1884). *J. Soc. Chem. Ind.* **3**, 297.
—— (1886). *J. Soc. Chem. Ind.* **5**, 218.
Votoček (1915). *Z. Zuckerind. Böhm.* **39**, 198.

XLVIII. NOTE ON DIHYDROXYACETONE.

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(Received January 31st, 1931.)

THE fact that dihydroxyacetone spontaneously polymerises [Levene and Walti, 1928] and also exists in two distinct crystalline modifications which are readily interconvertible [Abderhalden, 1911] makes it a matter of difficulty to prepare a consistently pure specimen. The commercial product as supplied by Meister, Lucius, Brüning and Co. under the name of "oxantin" which has been extensively used, gave variable results when tested physiologically, and we have shown [Reeves and Renbom, 1929] that on standing it becomes sticky and gives rise to appreciable quantities of lactic acid. The following typical treatment of the impure material gives a pure and stable compound.

EXPERIMENTAL.

Fifty grams of the commercial product were suspended in either 100 cc. of absolute alcohol, or better, pure acetone and shaken continuously for 24 hours at room temperature. After filtering and washing with the solvent, the precipitate was dried in a vacuum over CaCl_2 for 2 days. The filtrate was bright yellow in colour and strongly acid (p_{H} 5.5) and after removal of the solvent gave a clear yellow sticky mass which contained, in addition to lactic acid, traces of methylglyoxal as reported by Bernhauer and Wolf [1929]. 25.65 g. (51.3 % yield) of precipitate were thus obtained. Recrystallised from hot absolute alcohol it gave long colourless prismatic plates, soluble in H_2O , sparingly soluble in organic media and melting indefinitely at $82-83.5^\circ$ to a colourless liquid. At the melting point a turbidity developed and re-solidification was difficult owing possibly to the occurrence of dissociation.

Analysis C 39.96 %, H 6.65 % (theoretical C 40 %, H 6.66 %); a cryoscopic determination of the molecular weight in aqueous solution gave $M = 130$ (theoretical for the bimolecular compound $M = 180$) similar to that obtained by Wohl [1898].

The crystals were further identified by preparing the phenylosazone, m.p. 132° . Hence the dihydroxyacetone obtained was the bimolecular α -modification. When distilled under reduced pressure (5-8 mm.) it gives a distillate which crystallises in colourless needles m.p. $68-71.5^\circ$ and is unimolecular as determined cryoscopically in aqueous solution, $M = 86$ (theoretical $M = 90$)¹. This

¹ Dr F. Dickens informs us that he has obtained a similar result from both the crude and the purified oxantin.

latter compound corresponds to that obtained by Fischer and Mildbrand [1924] and is evidently the β -modification.

The authors wish to express their thanks to the Royal Society for a personal grant to one of them (H.G.R.) which has defrayed the cost of this work.

REFERENCES.

- Abderhalden (1911). *Biochem. Lexikon*, **2**, 270.
Bernhauer and Wolf (1929). *Biochem. Z.* **212**, 443.
Fischer and Mildbrand (1924). *Ber. deutsch. chem. Ges.* **57**, 707.
Levene and Walti (1928). *J. Biol. Chem.* **78**, 23.
Reeves and Renbom (1929). *Chem. Ind.* **48**; *Proc. Biochem. Soc.* Dec. 13th.
Wohl (1898). *Ber. deutsch. chem. Ges.* **31**, 2394.

XLIX. THE EFFECT OF ARGININE UPON THE BODY WEIGHT OF MICE INJECTED WITH THYROXINE, AND BEARING THE TUMOUR M 63.

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(Received February 3rd, 1931.)

IN an earlier paper [Gilroy, 1930, 2], the hypothesis was advanced that thyroxine might modify the growth rate of neoplastic tissue, and in the case of a transplantable tumour of the mouse, M 63. this assumption was to a great extent confirmed. The suggestion that thyroxine exerted an inhibitory effect by inducing the catabolism of tissue protein also received support, for it was possible to cancel the effect characteristic of thyroxine if arginine was administered during the same experimental period. the results suggesting the maintenance of normal equilibrium in relation to supply and demand.

It remained to be decided whether the action of thyroxine was primarily upon the tumour, or whether the latter was only affected indirectly owing to some alteration of the metabolism of the host. The injection of 0.1 mg. of thyroxine daily for 12 days did not induce any toxic effects, but when the experiment was extended to 18 days 7 mice died out of a total of 47, from which it appeared that the threshold of toxicity had then been reached.

The records of weight showed an average gain which corresponded very closely with increase in tumour mass, and there was no evidence of any loss of body weight, although it seemed probable that some reduction in the latter would have occurred. The work of Abderhalden and Wertheimer [1928], and Romeis [1923], has shown that in spite of the relative insusceptibility of rats and mice towards thyroxine, a loss of weight is induced by the repeated administration of small amounts. It was evident that if total weight remained stationary, or diminished, while the tumour continued to grow, a reduction in body weight must have occurred. Observations were therefore confined to a determination of the number of mice bearing tumours of increasing size in which total weight either remained stationary or diminished.

EXPERIMENTAL.

The technique of implantation was described in an earlier paper [Gilroy, 1930, 1]. Treatment consisted in the daily injection of 0.1 cc. of a 0.1 % solution of thyroxine, or of 0.3 cc. of a 10 % solution of arginine; in one group

both substances were injected, but the arginine was injected in the morning and the thyroxine at night. The total duration of the experiment was 12 days, the first injections being given 10 days after implantation; in certain groups treatment was reversed halfway through the experimental period, and the data are therefore given in every case for the 16th and 22nd days after implantation.

Table I shows the number of mice in which weight was gained, remained stationary, or diminished, expressed as a percentage, the actual numbers being given in brackets below.

Table I.

No. of mice	Weight increased		Weight stationary		Weight decreased	
	Day 16	Day 22	Day 16	Day 22	Day 16	Day 22
27	Arginine	Thyroxine	Arginine	Thyroxine	Arginine	Thyroxine
	89.0 (24)	55.6 (15)	7.4 (2)	22.2 (6)	3.7 (1)	22.2 (6)
27	Thyroxine	Arginine	Thyroxine	Arginine	Thyroxine	Arginine
	55.6 (15)	89.0 (24)	14.8 (4)	7.4 (2)	29.6 (8)	3.7 (1)
16	Arginine + thyroxine		Arginine + thyroxine		Arginine + thyroxine	
	87.5 (14)	94.0 (15)	— (0)	6.0 (1)	12.5 (2)	— (0)
18	Arginine	Arginine	Arginine	Arginine	Arginine	Arginine
	89.0 (16)	94.5 (17)	— (0)	— (0)	11.0 (2)	5.5 (1)
18	Thyroxine	Thyroxine	Thyroxine	Thyroxine	Thyroxine	Thyroxine
	72.2 (13)	83.5 (15)	— (0)	5.5 (1)	27.8 (5)	11.0 (2)
20	Untreated	Untreated	Untreated	Untreated	Untreated	Untreated
	95.0 (19)	75.0 (15)	— (0)	10.0 (2)	5.0 (1)	15.0 (3)

Owing to the small numbers involved no statistical significance can be attached to the results, but from the data presented certain inferences may be made. In a group of 27 mice injected with thyroxine for 6 days, 12 lost weight, while of 27 mice injected with arginine only 3 lost weight in the same number of days. Among 18 mice 8 lost weight when treatment with thyroxine was continued for 12 days and again only 3 out of 18 mice lost weight when injected with arginine for a similar period of time. When both arginine and thyroxine were injected for 12 days 3 out of 16 mice lost weight; this compares favourably with a loss of weight in 6 out of 20 in the group receiving no treatment at all.

Under ordinary circumstances, with normal stock, the injection of arginine does not appear to have any effect upon the body weight of mice, but it seems that if for any reason an undue strain is thrown upon the system of the animal, the tendency to lose weight can be compensated by the administration of this amino-acid. Thus the growth of a tumour absorbs certain nutritive substances at the expense of the tissues of the host; thyroxine increases the

demand for such materials by increasing general metabolism, and in both cases arginine seems capable of balancing the tissue loss. It may be inferred that arginine either exerts some protective action against the influence of thyroxine, or facilitates the immediate reconstruction of tissue protein.

The substances which are known to modify the effect of thyroxine upon metabolism fall into two groups; the first of these, exemplified by ergotamine [Abderhalden and Wertheimer, 1927], may be described as specific in their action; the second are non-specific and are represented by the carbohydrates. Arginine, being an inert substance, is clearly not to be included in the first category but it is possible that in the present instance arginine functioned as a precursor of sugar. That this might occur is shown by the studies of amino-acid metabolism in diabetes, from the results of which Dakin [1923], concluded that "arginine is the only amino-acid with more than five carbon atoms which can furnish glucose freely," the intermediate step being the formation of ornithine by the action of the enzyme arginase. It is therefore possible that under certain circumstances arginine may be glycogenic, and it is well known that a diet rich in sugar-forming materials can counteract the influence of thyroxine upon metabolism [Abderhalden and Wertheimer, 1926], while protein accentuates its effects.

Although arginine may serve as a source of sugar to the organism if need arises, this does not elucidate its action in accelerating the rate of tumour growth. It was shown by Cioffari and Piccaluga [1926], that a diet rich in carbohydrate did not have a stimulating effect, and in a previous series of experiments [Gilroy, 1930, 1], it was observed that amino-acids such as glycine and alanine were without any influence in increasing the rate of tumour growth, although these can be completely converted into glucose in the diabetic organism [Ringer and Lusk, 1910]. That arginine is an important constituent of tumours was shown by Kocher [1915], who found that it accounted for 12.4 % of the total protein nitrogen of neoplastic tissue. The specific importance of arginine in this connection was disputed by Drummond [1916], who pointed out that the diamino-acid content of any tissue was correlated with its nuclear content, and not with its rate of growth. It would seem, however, that the formation of nuclear material must involve the assimilation, or synthesis, of those substances which enter into its constitution, otherwise growth by cell division could not occur.

The alleged indispensability of arginine in general metabolism was questioned by Rose and Cox [1924], and Rose and Cook [1925], but more recently Scull and Rose [1930], have demonstrated in the case of rats the possibility of a synthesis of arginine in the body. It seems not unlikely that a synthetic mechanism may be provided by a reversal of the normal hydrolytic action of arginase, for it is known, from the experiments of Wasteneys and Borsook [1925], that proteolytic enzymes are also capable of synthesis. The high arginine content of tumours may perhaps be thus explained, since arginase has been shown by Edlbacher and Merz [1927], to be present in neoplastic

tissue in considerable amount. A tissue thus equipped would not be dependent upon preformed arginine for nuclear synthesis and would to this extent more readily attain optimum conditions for growth.

SUMMARY.

126 mice were implanted with the carcinoma M 63, and 10 days later were divided into groups, 88 mice receiving injections of thyroxine in doses of 0.1 mg. daily for a period of 6 or 12 days. In certain groups arginine was injected coincidentally with thyroxine; the remainder were either injected with arginine alone, or were given no treatment at all.

The mice were weighed individually three times during the experiment and the results obtained indicated that arginine prevented a loss of weight which thyroxine would otherwise have induced.

As a high carbohydrate diet is known to modify the influence of thyroxine upon metabolism it is suggested that, in this instance, arginine was utilised for the formation of sugar, the latter being derived from ornithine, but reasons are advanced for considering that the glycogenic function of arginine could not be responsible for increasing the rate of tumour growth.

I wish to record my indebtedness to the Medical Research Council for a grant which made this investigation possible, and to Professor Crew for scientific hospitality, and for his encouragement and advice.

REFERENCES.

- Abderhalden and Wertheimer (1926). *Pflüger's Arch.* **213**, 328.
——— (1927). *Pflüger's Arch.* **216**, 697.
——— (1928). *Pflüger's Arch.* **219**, 594.
Cioffari and Piccaluga (1926). *Tumori*, **12**, 387.
Dakin (1923). *Oxidations and reductions in the animal body*, pp. 64–71 (London, 2nd edition).
Drummond (1916). *Biochem. J.* **10**, 473.
Edlbacher and Merz (1927). *Z. physiol. Chem.* **171**, 251.
Gilroy (1930, 1). *Biochem. J.* **24**, 589.
——— (1930, 2). *Biochem. J.* **24**, 1181.
Kocher (1915). *J. Biol. Chem.* **22**, 295.
Ringer and Lusk (1910). *Z. physiol. Chem.* **66**, 106. Cited by Mitchell and Hamilton, *The biochemistry of the amino-acids* (1929).
Romeis (1923). *Biochem. Z.* **135**, 85; *Amer. Chem. Abst.* **17**, 2751.
Rose and Cox (1924). *J. Biol. Chem.* **61**, 747.
——— and Cook (1925). *J. Biol. Chem.* **64**, 325.
Scully and Rose (1930). *J. Biol. Chem.* **89**, 109.
Wasteneys and Borsook (1925). *J. Biol. Chem.* **62**, 633, 675; **63**, 563.

L. IS AVITAMINOSIS B₁ AN INTOXICATION BY METHYLGLYOXAL?

GLYOXALASE—CO-ENZYME RATIO IN EXPERIMENTAL BERIBERI.

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(Preliminary Communication.)

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IN 1921 Findlay [1921] reported that "the glyoxalase content of the liver in pigeons with beriberi is less than that in control pigeons," and "the administration of vitamin B to a beriberic pigeon is followed by an increase in the glyoxalase content of the liver," but "vitamin B does not act as a co-enzyme of glyoxalase." Findlay drew no conclusion concerning the nature of the toxic substance supposed to come into existence during carbohydrate metabolism in the absence of the antineuritic vitamin. Since Findlay's discovery important investigations have been made both with respect to vitamin B and to the conditions of dismutation of methylglyoxal (and phenylglyoxal).

The complex nature of water-soluble vitamin B, viz. its resolution into the components B₁ (antineuritic and growth-promoting vitamin) and B₂ (P.P. factor, growth-promoting and pellagra-preventing vitamin) is now a well-known fact; it is possible that more components are contained in the vitamin B complex, but as yet this has not been definitely elucidated.

According to our present knowledge vitamin B₁ seems to be closely related to carbohydrate metabolism [Evans and Lepkovsky, 1929-30] and, through the latter, possibly also to protein metabolism. As to the importance of vitamin B₁ for fat metabolism it can only be said to be minimal; as yet the importance of vitamin B₂ in metabolism is unknown.

Several investigators have spoken in favour of the toxic pathogenesis of beriberi, none of them, however, stating the nature of the toxic substance with certainty; it was only supposed to come into existence during carbohydrate metabolism in the absence of vitamin B₁. The hypothesis most recently advanced [Peters, 1930], associated avitaminosis B₁ with an increase of the amount of lactic acid in the organism, especially of the brain. Peters believes that vitamin B₁ is concerned with the removal of lactic acid in the Meyerhof carbohydrate cycle.

As far as I know the literature does not comprise any work associating beriberi with an intoxication by methylglyoxal.

As demonstrated by Toenniessen and Fischer [1926], Ariyama [1928, 1, 2] and Vogt [1929] methylglyoxal is, also in animal glycolysis, an intermediary substance in carbohydrate catabolism, a dismutation of methylglyoxal into lactic acid eventually taking place.

Formerly methylglyoxal was considered a non-toxic substance [Dakin and Dudley, 1913, 4] but this view proved to be erroneous; it is a toxic substance as shown by Sjollem and Seekles [1926], Fischler [1927], Kermack, Lambie and Slater [1927], and Herring and Hynd [1928].

Normally methylglyoxal cannot be demonstrated in the organism, a dismutation of the substance by means of glyoxalase (ketonealdehydemutase) taking place in the presence of the co-enzyme (co-mutase). Neuberger and Kobel [1928, 1929, 1, 2] noticed that well-washed lactobacillus or yeast, which was capable of converting hexosediphosphate to methylglyoxal but incapable of forming lactic acid from methylglyoxal, acquired the ability of producing lactic acid when boiled yeast juice was added to it. This was interpreted by them as indicating that co-enzyme was indispensable for the enzymic production of lactic acid from methylglyoxal. It was also demonstrated by Vogt [1929] in the case of liver (and other organic) enzyme systems that no dismutation of methylglyoxal will take place in the absence of the co-enzyme; under these conditions methylglyoxal is stabilised as such, and dismutation will only take place when co-enzyme is added to the enzyme system. Glyoxalase (a much better, because more logical, term would be ketonealdehydemutase as suggested by Neuberger [1913, 1, 2]) is widely distributed in nearly all tissues, more especially in the liver and muscles; and besides its rôle in the metabolism of sugar it also plays a part in the conversion of *D*-alanine to lactic acid. The presence of glyoxalase in the tissues is thus of importance in relation to the metabolism of carbohydrates and proteins. The wide distribution of glyoxalase in the plant and animal worlds seems to afford a strong indirect support for the methylglyoxal theory.

In my experiments mice and pigeons were used and three different methods employed. (1) The technique of Dakin and Dudley [1913, 1, 2, 3] and Neuberger [1913, 1, 2] which consists essentially in allowing a 20 % watery extract of the tissues to act on methylglyoxal (phenylglyoxal), and when the methylglyoxal (phenylglyoxal) is converted into lactic acid (mandelic acid), extracting the acid and estimating it by titration in terms of *N*/10 NaOH. (2) The technique of Vogt (1929) which consists in precipitation of methylglyoxal with 2 : 4-dinitrophenylhydrazine as bis-hydrazone. (3) The colorimetric method introduced by Ariyama [1928, 1] which is based upon the fact that glyoxals acquire, by the addition of cyanide, such a great increase of reducing intensity in alkaline solution that they cause the colour development of Benedict's arsenophosphotungstic reagent. None of the possible intermediate products of sugar metabolism, such as formic, acetic, glycollic, and glyceric aldehydes,

lactic and pyruvic acids or free hexose, interferes with the determination by developing the blue colour, with the exception of dihydroxyacetone, which gives about 20 % of the colour of methylglyoxal of the same concentration.

I thus succeeded in proving that methylglyoxal cannot be demonstrated in hexosephosphate + liver enzyme systems in starved animals any more than in normal animals, but that this substance can be demonstrated in hexosephosphate + liver extract from animals that do not get vitamin B₁, *i.e.* in animals otherwise getting a complete diet, comprising vitamins A + D (cod-liver oil), B₂ (autoclaved yeast), and E (wheat germ oil). I succeeded in demonstrating that the ingestion of vitamin B₁ (tikitiki-extract or Peter's concentrate) by the animals experimented upon apparently removed the difficulty attending the dismutation of methylglyoxal into lactic acid, and I also succeeded in demonstrating that in liver enzyme systems from animals suffering from avitaminosis B₁ methylglyoxal (and phenylglyoxal) is only converted into lactic acid (mandelic acid) to a small extent, whilst it was impossible in liver enzyme systems originating from normal (or starved) animals to demonstrate the presence of added methylglyoxal after the mixture had been allowed to stand for 24 hours. According to our present knowledge the enzyme is present in the liver tissue of animals suffering from avitaminosis B₁, whilst it seems to be the co-enzyme that is lacking, as a boiled aqueous extract of a normal animal liver, when added to the enzyme system originating from animals suffering from avitaminosis B₁, brings about the dismutation. This proof however is not conclusive when considered in relation to Vogt's [1929] investigations, because aqueous boiled extract of liver is also capable, although to a small extent, of bringing about a dismutation of methylglyoxal, the more so as the experiments with co-enzyme originating from yeast did not give completely satisfactory results (compare Findlay's results [1921]). As yet it cannot be decided with certainty whether it is the enzyme or, more probably, the co-enzyme that is lacking; perhaps there is a change of concentration in the enzyme/co-enzyme ratio of the organs of animals suffering from beriberi.

The fact that there is a possibility of accumulating methylglyoxal in animals suffering from avitaminosis B₁ is the more interesting as the symptoms of methylglyoxal intoxication in many respects bear a resemblance to those of experimental beriberi. Possibly the low blood-sugar level in the terminal stage of avitaminosis B₁ plays a role in regard to the sensitiveness of the animal to methylglyoxal; it is interesting in this connection to note that polyneuritic symptoms in the mouse resemble insulin symptoms in the same animal, especially interesting because methylglyoxal possibly plays a part in the production of the symptoms of insulin hypoglycaemia.

The whole of the experimental material forming the basis of this preliminary communication will soon be published.

SUMMARY.

The hypothesis is set up that the symptoms of avitaminosis B₁ are caused, wholly or partly, by an intoxication by methylglyoxal occurring on account of a failure of the dismutation of methylglyoxal, the tissue being deprived of the co-enzyme (not glyoxalase itself), or at least of a greater quantity of co-enzyme in comparison with glyoxalase.

REFERENCES.

- Ariyama (1928, 1). *J. Biol. Chem.* **77**, 359.
— (1928, 2). *J. Biol. Chem.* **77**, 395.
Dakin and Dudley (1913, 1). *J. Biol. Chem.* **14**, 155.
— — (1913, 2). *J. Biol. Chem.* **14**, 423.
— — (1913, 3). *J. Biol. Chem.* **15**, 463.
— — (1913, 4). *J. Biol. Chem.* **15**, 127.
Evans and Lepkovsky (1929–30). *J. Nutrit.* **2**, 1.
Findlay (1921). *Biochem. J.* **15**, 104.
Fischler (1927). *Z. physiol. Chem.* **165**, 68.
Herring and Hynd (1928). *J. Physiol.* **66**, 267.
Kermack, Lambie and Slater (1927). *Biochem. J.* **21**, 40.
Neuberg (1913, 1). *Biochem. Z.* **49**, 502.
— (1913, 2). *Biochem. Z.* **51**, 484.
— and Kobel (1928). *Biochem. Z.* **203**, 463.
— — (1929, 1). *Biochem. Z.* **207**, 232.
— — (1929, 2). *Biochem. Z.* **210**, 466.
Peters (1930). *J. State Med.* **38**, 63.
Sjolleme and Seekles (1926). *Biochem. Z.* **176**, 431.
Toenniessen and Fischer (1926). *Z. physiol. Chem.* **161**, 254.
Vogt (1929). *Biochem. Z.* **211**, 17.

LI. THE INACTIVATION OF TRYPSIN-KINASE BY HEAT, AND THE EFFECT OF ADDED PROTEIN THEREON.

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(Received February 6th, 1931.)

IN previous papers the writer has described experiments on the inactivation of trypsin [1930] and of enterokinase [1931] by heat. The first section of the present paper deals with the inactivation by heat of trypsin-kinase, the complex formed by union of the enzyme trypsin with its specific activator enterokinase. The examination of the heat-inactivation of trypsin-kinase involves in principle the three following operations:

- (1) the formation of trypsin-kinase by mixing solutions of trypsin and enterokinase and leaving the mixture for 30 mins. in a thermostat at 30°;
- (2) the transference of the trypsin-kinase, resulting after the above period of activation, to a thermostat at 50°, and the removal of samples at various time intervals;
- (3) the measurement of the trypsin-kinase content of the various samples by the extent of the hydrolysis produced in a caseinogen solution under standard conditions.

The general experimental procedure involved in these operations may now be briefly described.

Experimental procedure.

For comparison with the work on the heat-inactivation of trypsin free from enterokinase, where the glycerol concentration was 24 %, it is necessary to arrange in the present investigation that the solution of trypsin-kinase undergoing inactivation should have the same glycerol concentration. This is arrived at in the following way.

5 g. of dried intestinal mucosa, prepared according to the method of Waldschmidt-Leitz [1924], were dispersed through 200 cc. of a glycerol-water mixture (containing 23.6 % of glycerol) by shaking and the dispersion was left for 24 hours in the ice-chest to allow of the activator passing into pseudo-solution. The dispersion was then centrifuged and filtered, and to 160 cc. of the filtrate were added 3 cc. of *N* acetic acid. The resulting precipitate of protein matter was centrifuged off, and the clear supernatant liquid neutralised

with *N* ammonia and kept in the ice-chest. This kinase solution now contained 23 % of glycerol. The experiments were then carried out as follows.

15 cc. of trypsin solution prepared as described in a previous paper of the writer [1930] (and containing 26.4 % of glycerol) were added to 10 cc. of the kinase solution described above. Formation of trypsin-kinase was allowed to occur by maintaining the mixed solutions for 30 mins. at 30°. At the end of this period 1 cc. of p_H -adjusting liquid (*N* acetic acid, *N* ammonia and distilled water) was added, thus producing in the final solution a glycerol content of 24 %. Of this final solution 3 cc. were measured out into each of a number of flasks already immersed in a thermostat at 50°. These flasks were withdrawn into crushed ice at various time intervals and the quantity of trypsin-kinase, *i.e.* the number of "units" therein, determined by the method of Linderstrøm-Lang and Steenberg [1929] at 30°, as described by the writer in a previous paper [1931].

The p_H measurements were carried out at room temperature by means of the glass electrode.

It was found that the course of the reaction, using this solution of trypsin-kinase, is in fair agreement with the unimolecular expression¹:

$$k = \frac{2.303}{t} \log_{10} \frac{a}{a-x}$$

where

a = initial content of trypsin-kinase,

$a - x$ = content of trypsin-kinase after time t mins.

Table I, which is typical of the results in general, illustrates the degree of agreement with the unimolecular expression.

Table I.

Temperature 50°. p_H 8.77. Glycerol 24 %.

Time (mins.)	Trypsin-kinase content units per cc.	$k \times 10^3$
0	1.00	—
30	0.83	6.21
60	0.67	6.67
100	0.54	6.16
220	0.25	6.30

The effect of p_H upon the heat-inactivation.

The stability of all enzymes is influenced very markedly by the p_H of the medium, and Table II shows the effect of p_H upon the heat-inactivation of trypsin-kinase. In the second column the mean of the values of k for a particular p_H are set down opposite that p_H . The third column shows "smoothed values" of k obtained from the most probable curve resulting on plotting the experimental values of k against p_H . This curve is shown in Fig. 1.

¹ Throughout this paper k represents the velocity constant of a unimolecular reaction.

Table II.

Temperature 50°. Glycerol 24 %.

p_H	$k \times 10^3$	
	Experimentally found	"Smoothed" from curve
4.10	4.9	4.9
4.20	4.7	4.7
4.97	3.3	3.4
5.09	3.3	3.2
5.90	2.1	2.3
5.98	2.4	2.2
6.50	1.9	2.0
6.57	2.2	2.05
7.88	3.4	3.5
8.15	4.6	4.3
8.67	6.6	6.3
8.77	6.3	6.9
8.92	9.6	9.6

It is seen that the optimum stability for trypsin-kinase lies about p_H 6.5.

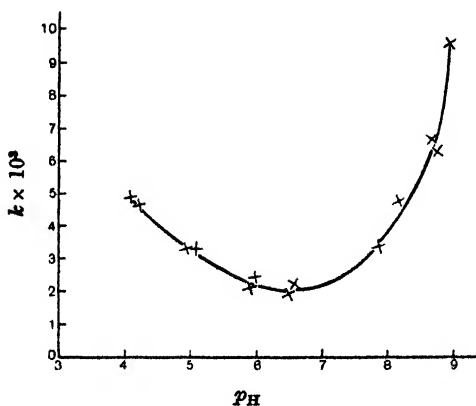


Fig. 1.

The critical increment for the heat-inactivation of trypsin-kinase.

Experiments were carried out to determine the critical increment for the heat-inactivation of trypsin-kinase. This was determined by measuring k at 50° and 60° and then substituting the mean values in the integrated form of the equation:

$$\frac{d \log_e k}{dT} = \frac{E}{RT^2}.$$

Measurements were made:

- (1) in the region of optimum stability, p_H 6.5,
- (2) in the acid region at p_H 4.1, p_H 5.1, p_H 5.9,
- (3) in the alkaline region at p_H 8.8.

The results are summarised in Table III in which the figures in the last column represent the critical increments calculated by substituting the mean values of k at 50° and 60° into the integrated form of the equation.

Table III.

p_H	k (at 50°) $\times 10^3$	k (at 60°) $\times 10^3$	E (cals.)
8.8	6.34	4.77	43,000
6.5	1.90	1.55	44,600
5.9	2.14	1.35	39,000
5.1	3.27	2.80	45,000
4.1	4.95	2.90	38,000

The critical increment appears to vary with p_H in a fortuitous manner. It may be concluded that over the p_H range 4–8.8 it is of the order of 40,000 calories per “molar unit” of trypsin-kinase.

Comparison with the results obtained for trypsin¹ free from enterokinase.

It will be seen, on comparing these results with those obtained by the writer [1930] for the heat-inactivation of trypsin free from enterokinase, that the behaviour is similar in two important respects:

- (1) in the position of the region of optimum stability, namely about p_H 6.5;
- (2) in the order of magnitude of the critical increment, namely about 40,000 calories, being sensibly the same at the region of optimum stability and at points on the acid and alkaline sides in each case.

This similarity suggests that the active enzymic groupings may still be of the same nature in trypsin-kinase as they are in trypsin. Since however trypsin-kinase has more extensive proteolytic activity than trypsin alone it is to be inferred that the attachment of the kinase, necessarily at points other than those proteolytically active, serves to intensify the latter.

A comparison of the actual rates of heat-inactivation in both cases is of interest. This may possibly be best effected if, instead of comparing unimolecular constants, we compare the times of “half decomposition.” This time interval is obtained from the unimolecular expression by writing

$$k = \frac{2.303}{t_{\frac{1}{2}}} \log_{10} 2.$$

The comparison is made in Table IV. The values of k corresponding to the

Table IV.

Temperature 50° . Glycerol 24 %.

p_H	Time for “half decomposition” (mins.)	
	Trypsin	Trypsin-kinase
5.0	231	210
6.0	346	277
7.0	346	300
8.0	247	193
8.5	198	128
9.0	144	74

¹ The word trypsin is used here in the sense employed by the Willstätter school up to 1930, i.e. the enzyme inactive towards true proteins but capable of hydrolysing certain protein degradation products. It has since been shown that trypsin itself may be resolved into a proteinase and a carboxy-polypeptidase. It is the latter fraction which is responsible for the hydrolysis of protein degradation products.

p_H - k curves for trypsin and trypsin-kinase respectively are calculated and then substituted into the equation for $t_{\frac{1}{2}}$ given above.

It is seen from Table IV that the rates of heat-inactivation of trypsin and of trypsin-kinase are fairly close together. On the whole it is to be concluded that trypsin is slightly more stable when free from its activator than when associated with it. This superior stability of trypsin over trypsin-kinase is most marked in the alkaline region.

*The effect of the addition of protein upon the heat-inactivation
of trypsin-kinase.*

Proteins, peptones and amino-acids have been found to have a stabilising influence upon a number of enzymes. Luers and Lorinser [1922] found that egg-albumin and gelatin have a stabilising influence upon malt amylase, and Willstätter, Graser and Kuhn [1922] showed that leucylglycine and yeast gum protect saccharase to some extent from heat-inactivation. When the proteins and their degradation products are the substrates and products of reaction respectively, as with the proteolytic enzymes, the stabilising effect is most pronounced, for enzymes generally are found to be protected by their specific substrates and their products of reaction. Thus Biernacki [1891] found that albumins protected trypsin from heat-inactivation, and Bayliss and Starling [1904] have observed a similar effect with caseinogen peptones and egg-albumin. Northrop [1922] has stated that the products formed by the action of trypsin on proteins have a protective action upon the enzyme, but that gelatin itself has no such stabilising action.

In the following experiments, carried out by the writer, caseinogen was added to the solution of trypsin-kinase undergoing inactivation at two different p_H values:

- (1) at p_H 8.5, i.e. in the region of optimum activity for the enzyme, where the products of reaction are most readily formed;
- (2) at p_H 4.7, i.e. well outside the optimum region of activity for the enzyme.

The experimental procedure was as follows.

At p_H 8.5. To 25 cc. of trypsin-kinase, prepared as described above, were added 2 cc. of a buffer, prepared by mixing equal volumes of N ammonia and N ammonium chloride, and 2 cc. of a 6 % caseinogen solution.

As a control the same solution was made up except that instead of 2 cc. of a 6 % caseinogen solution, 2 cc. of distilled water were used.

At p_H 4.7. The same solution and control were made up as at p_H 8.5 except that 2 cc. of a buffer prepared by mixing equal volumes of 0.066 N sodium acetate and 0.066 N acetic acid were used instead of the alkaline buffer.

The solutions containing the added protein and also the control solutions were maintained at 50° for 90 mins. and the trypsin-kinase content was measured initially and at the end of this period of time. The results are shown in Tables V and VI.

Table V.

Temperature 50°. p_H 8.5.

	Time (mins.)	Trypsin-kinase units per cc.		$k \times 10^3$
		Run A	Run B	
With added protein	0	1.06	1.00	Run A 3.3
	90	0.76	0.73	Run B 3.2
Without added protein	0	1.06	1.01	Run A 5.8
	90	0.63	0.61	Run B 5.6

From these results it follows that the mean decrease in k due to the added protein is 43 %.

Table VI.

Temperature 50°. p_H 4.7.

	Time (mins.)	Trypsin-kinase units per cc.	
		Run A	Run B
With added protein	0	1.09	1.05
	90	0.85	0.81
Without added protein	0	1.09	1.05
	90	0.83	0.80

It is seen that at this p_H of 4.7 the difference between the degree of inactivation of the solution containing added protein and that of the control is within the experimental error.

It is to be concluded from these experiments that it is the products of hydrolysis which exert a stabilising influence upon the enzyme, the protein as such having little effect.

A further experiment was carried out in which the heat-inactivation was again determined at the alkaline p_H 8.5, but at 60° instead of at 50°. Since the critical increment for the tryptic hydrolysis of caseinogen is about 14,000 calories [Moelwyn-Hughes, Pace and Lewis, 1930] while the critical increment for the heat-inactivation of the enzyme is seen to be of the order of 40,000 calories, a rise of 10° in temperature will result in a relatively smaller increase in the velocity of hydrolysis as compared with the increase in the velocity of inactivation. So that if, as we have inferred, the products of hydrolysis exert a stabilising influence upon the enzyme, this effect should be relatively less at 60° than it is at 50°. The results of the experiment are seen in Table VII.

Table VII.

Temperature 60°. p_H 8.5.

	Time (mins.)	Trypsin-kinase units per cc.	$k \times 10^3$
With added protein	0	1.02	3.6
	90	0.71	
Without added protein	0	1.02	4.0
	90	0.68	

The decrease in k owing to added protein is now 10 %, compared with the decrease of 43 % in the corresponding experiment at 50°. This result is therefore in agreement with the conclusion that it is the products of reaction rather than the protein itself which stabilise the enzyme to some extent against heat-inactivation.

In experiments carried out by the writer on the heat-inactivation of a sample of commercial "active trypsin" (*i.e.* trypsin-kinase) it was found that the constants calculated on the basis of the unimolecular expression decreased with increase in time. Northrop [1922] found the same behaviour in experiments with commercial trypsin. He put forward the explanation that the slowing up of the reaction is due to the enzyme acting upon the protein matter in the preparation used, when the resulting products of hydrolysis unite with the enzyme to form compounds which are more stable to heat than the free enzyme. This conclusion, arrived at by Northrop, is substantially confirmed by the results described in this paper.

SUMMARY.

1. A solution of trypsin-kinase has been prepared by the interaction of trypsin and enterokinase solutions for 30 mins. at 30°. The solution was used to study the heat-inactivation of trypsin-kinase. At the degree of purity employed it is found that the course of the reaction is in fair agreement with the unimolecular expression.

2. The effect of p_H upon the heat-inactivation has been investigated and it is found that the optimum stability is about p_H 6.5.

3. The critical increment of the heat-inactivation process has been determined at five p_H values, lying between p_H 4 and 9. It is found that the critical increment is of the order of 40,000 calories per "molar unit" of enzyme.

4. Experiments have been carried out upon the effect of adding caseinogen to the solution of trypsin-kinase undergoing inactivation. The caseinogen was added to solutions of the enzyme at p_H 8.5 (in the optimum region of activity for trypsin-kinase) and at p_H 4.7 (well outside the optimum region of activity) respectively. It was found that at p_H 8.5 the effect of the caseinogen, added initially, is to slow up the heat-inactivation of the enzyme, but that at p_H 4.7 there is no marked stabilisation. Thus it is concluded that it is the products of hydrolysis of the caseinogen which stabilise the enzyme. Further support for this conclusion was obtained by conducting the heat-inactivation of the enzyme, with caseinogen added initially, again at p_H 8.5 but at a temperature 10° higher. It was found that under these conditions the relative stabilising effect is reduced.

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REFERENCES.

- Bayliss and Starling (1904). *J. Physiol.* **30**, 61.
Biernacki (1891). *Z. Biol.* **28**, 49.
Linderstrøm-Lang and Steenberg (1929). *Compt. Rend. Trav. Lab. Carlsberg*, **17**, No. 16.
Luers and Lorinser (1922). *Biochem. Z.* **133**, 487.
Moelwyn-Hughes, Pace and Lewis (1930). *J. Gen. Physiol.* **13**, 323.
Northrop (1922). *J. Gen. Physiol.* **4**, 261.
Pace (1930). *Biochem. J.* **24**, 606.
—— (1931). *Biochem. J.* **25**, 1.
Waldschmidt-Leitz (1924). *Z. physiol. Chem.* **132**, 181.
Willstätter, Graser and Kuhn (1922). *Z. physiol. Chem.* **123**, 1.

LII. THE MOLECULAR WEIGHT OF VITAMIN A.

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INTRODUCTION.

RECENT experiments on the problem of the relation between carotene and vitamin A bore evidence in favour of the view that in the animal body carotene is being transformed into vitamin A [Moore, 1929; Capper, 1930; Wolff *et al.* 1930]. This being so, carotene would be the vegetable source from which vitamin A is built up and should be considered as a "provitamin." An artificial conversion has not yet been obtained. Euler, Euler and Karrer [1929] have suggested that vitamin A might be a partly hydrogenated carotene, but no evidence has been found about the nature of their chemical relation.

It seemed important to us to determine the relative molecular size of the two substances. We therefore determined their diffusion constants in the same solvent. Were these constants found to be of almost the same order of magnitude, then the conclusion could be drawn that a simple chemical relation (*e.g.* a hydrogenation or an oxidation) existed.

However, the result was the reverse. The diffusion constants of carotene and vitamin A showed such a difference that their chemical relation cannot be a simple one. If the idea that one substance is converted into the other is right, the conversion cannot consist in simple hydrogenation, but must be a more radical transformation of the molecule. It must be stated here, that what we considered as "vitamin A," was determined by the reaction of Carr and Price (the blue colour developed by a solution of SbCl_3 in chloroform). We started from the point of view that up to now there has been evidence enough to consider this reaction as specific for the substance with vitamin properties when the absorption band is at 610μ .

In this connection we should like once more to draw attention to the fact that the transformation *in vivo* was also detected by the increase of the blue colouring capacity of liver extracts after animals had been fed with carotene.

Materials.

Vitamin A preparations were obtained from the unsaponifiable part of sheep-liver extracts. After saponification, the light petroleum extract was concentrated, cooled and filtered. The solution now contained about 0.1 mg.

of solid *per* rat unit of vitamin A¹ and was by no means a pure vitamin solution as the actual active constituent was perhaps 2 % of the solid residue. Since indifferent substances do not appreciably influence each other's rates of diffusion provided that their solutions are not too concentrated, it seemed possible to work with these impure preparations. By evaporating the light petroleum *in vacuo* in a carbon dioxide atmosphere, adding xylene and evaporating half of the latter, a clear solution in xylene was obtained. Crystalline carotene was prepared from carrots and showed a m.p. of 174°. Considering the desirability of avoiding convection currents, when bringing the pure solvent above the very dilute carotene solution in order to effect diffusion, it was necessary to bring the solution to a higher specific gravity than that of the pure solvent. Therefore a certain amount of cholesterol was added. This amount was in Exps. 1 and 2 about 6 %, in Exps. 3 and 4 about 3 %.

The ratio of carotene to the total solid was in Exp. 2 1:630, in Exp. 3 1:60. The solutions of carotene in xylene proved extremely unstable, even when kept hermetically closed in a dark room. To get rid of this inconvenience in the second series of experiments a small quantity of pyrogallol was added to the solution which proved to be very effective in keeping the quantity of carotene unchanged for a considerable time if preserved in the dark.

Method of measuring the diffusion velocity.

In our experiments we used the diffusion apparatus of Cohen and Bruins [1923]. This consists of six glass discs, which may be rotated around a common axis, all being of exactly the same thickness. With the exception of the lowest and uppermost, all the discs are provided with three round perforations fitting exactly upon each other. When the discs are put together these perforations make three cylindrical spaces. The actual experiment is carried out by filling the lowest section of these cylinders with the solution to be investigated and the three higher compartments with the pure solvent. By suitably and very carefully rotating the discs, the upper three compartments are now brought above the lower one. After the diffusion has taken place for a certain time, the four compartments are again separated by rotating two alternate discs (Nos. 2 and 4). In this way the column of liquid is divided into four equal parts. Every one of these parts is analysed separately. From the ratio of the concentrations of the dissolved substance in the four layers the diffusion constant may be calculated. For a detailed description of the apparatus and of the calculation the original literature should be consulted.

A few simplifications of the original procedure were introduced². As our analytical methods did not permit a very high exactitude, part of the precautions usually exercised in diffusion experiments were not considered im-

¹ We have to thank N. V. Organon, Oss, for having supplied us with this preparation.

² The diffusion apparatus was placed in the cellar of the van 't Hoff Laboratory, Utrecht (Director Prof. Dr E. Cohen).

perative in this case. In the first series of experiments, with both substances, we did not work with a thermostat, but wrapped the diffusimeter in cotton wool and took care that no sudden changes of temperature should occur. During the second series the apparatus was placed in the air-thermostat [Cohen and Bruins, 1923, p. 371], but the water in the thermostat did not circulate and therefore no auxiliary thermostat was needed. The rotation of the discs was not performed mechanically but very carefully by hand. Before starting the diffusion process, the apparatus was kept for 3 hours in the thermostat.

In all our experiments we used pure *m*-xylene (B.P. 139–139.5°) as a solvent. The discs were lubricated with glycerol in the first series. As this took up moisture from the air very easily, the edges were greased with soft soap. In the second series the discs were lubricated by a hydrogel of glycerol-amylum.

Calculation.

Each diffusion experiment gave us three concentration values for each cylindrical section, the average of which was used for the calculation of the diffusion constant. To this end the tables of Kawalki [1894] were used.

Intermediate values, not recorded in these tables, were found by linear interpolation. In this way we obtained from each experiment four values of x in the formula:

$$x = \frac{h'^2}{D \cdot t}$$

where h' is the half of the height of one section, D is the diffusion constant, t is the time of diffusion. From these four values the average was calculated for each diffusion-experiment, their relative "weights" being taken into account [for details see Cohen and Bruins, 1923].

The time of diffusion in all experiments was about the same, hence the following values for the "weight" p of the various sections were used, the relative variations in the concentrations of the four sections being between $x = 0.1296$ and $x = 0.1936$, i.e. the part of the tables used in these calculations.

Section 1	$p = 529$
2	$p = 312$
3	$p = 178$
4	$p = 660$

$$\overline{p} = 1679 \quad x_{av} = \frac{\sum px}{\sum p}$$

The diffusion constant is found from the formula:

$$D_{av} = \frac{h'^2}{x_{av} \cdot t}$$

For our diffusimeter $h'^2 = 0.2376$ [Cohen and Bruins, 1924], D is expressed in cm.² per 24 hours.

For the calculation of the diffusion constant at 20° we used a temperature

coefficient of 1.4 % per ° C. This is the temperature coefficient of the viscosity of *m*-xylene calculated from the data given by Thorpe and Rodger (Landolt-Börnstein tables).

Method of analysis.

In these diffusion experiments only the relative concentrations in single sections are to be determined, and it is not required to know the absolute concentration of the substance investigated. This is very important for the reason that only in view of this fact may we use without any objection the reaction of Carr and Price [1926] for our vitamin A determinations. However doubtful the absolute value of this reaction is, the units by which we express the concentration value of the cylindrical sections may be quite arbitrary.

A. Vitamin A determination.

The reaction was carried out in the usual way (0.2 cc. of the solution, to which two drops of acetic anhydride were added to prevent precipitation, were mixed with 2 cc. of a saturated solution of SbCl_3 in chloroform [Bertram, 1929], and the blue colour measured in the Lovibond colorimeter.

The colour expressed in Lovibond units not being a linear function of the dilution, a standard curve of this function was made.

The solutions to be investigated were always brought to such dilutions that the reaction of Carr and Price gave a blue colour corresponding to about 5 units. Two other dilutions were made, if possible of about 4 and 6 units. From these three determinations the average value was obtained. The values recorded in the Tables are these averages calculated in rat units of Rosenheim per cc. solution (5 Lovibond units are equal to 5 rat units). As already stated, the absolute value of the units is of no importance. The error in our series of experiments amounts to about 5–10 %. Errors of this range of magnitude seemed rather disturbing for the reliability of the diffusion constants to be calculated; therefore the average of three concentration measurements was used in these calculations.

B. Carotene determinations.

The determinations of carotene were carried out colorimetrically with the stuphometer of Zeiss, by means of a standard curve established in a preliminary series with solutions of known concentration. Carotene having an absorption band at 490–475 $\mu\mu$ the filter S_{47} was used in these determinations. Solutions of carotene containing 0.001–0.01 mg. per cc. can be determined very exactly with the photometer. The probable error in these estimations does not exceed 3 %. In the following tables the concentrations are recorded in 0.001 mg. per cc. These figures also represent the average of three determinations.

RESULTS OF THE EXPERIMENTS.

Vitamin A; first series.

I.

Diffusion time: 3.030 days; temp. 16.3°–15.0°; average 15.7°.

	A	B	C	Average	0/000	x	$p \cdot x$	
1	73	—	72	72	1109	0.1477	78.13	$x_{av} = \frac{244.26}{1679} = 0.1455$
2	127	—	123	125	1926	0.1397	43.59	
3	188	—	193	191	2943	0.1226	21.82	
4	269	—	253	261	4021	0.1526	100.72	
				649	9999		244.26	

 $D = 0.540 \text{ cm.}^2/24 \text{ hours; } D_{20} = 0.572 \text{ cm.}^2/24 \text{ hours.}$

II.

Diffusion time: 3.018 days; temp. 16.1°–18.2°; average 17.2°.

	A	B	C	Average	0/000	x	$p \cdot x$	
1	—	47	48	47.5	1237	0.1343	71.05	$x_{av} = \frac{248.18}{1679} = 0.1478$
2	—	77	62	69.5	1811	0.1613	50.33	
3	—	111	102	106.5	2773	0.0835	14.86	
4	—	157	164	160.5	4180	0.1696	111.94	
				384.0	10001		248.18	

 $D = 0.531 \text{ cm.}^2/24 \text{ hours; } D_{20} = 0.552 \text{ cm.}^2/24 \text{ hours.}$

III.

Diffusion time: 3.015 days; temp. 18.9°–19.5°; average 19.2°.

	A	B	C	Average	0/000	x	$p \cdot x$	
1	58	58	—	58	1090	0.1495	79.09	$x_{av} = \frac{235.16}{1679} = 0.1401$
2	107	107	—	107	2012	0.1222	38.13	
3	158	157	—	158	2971	0.1242	22.11	
4	208	210	—	209	3929	0.1452	95.83	
				532	10002		235.16	

 $D = 0.564 \text{ cm.}^2/24 \text{ hours; } D_{20} = 0.570 \text{ cm.}^2/24 \text{ hours.}$ *Vitamin A; second series.*

I.

Diffusion time: 2.950 days; temp. 19.3°–19.7°; average 19.5°.

	A	B	C	Average	0/000	x	$p \cdot x$	
1	33	32	34	33.0	1235	0.1347	71.26	$x_{av} = \frac{224.89}{1679} = 0.1339$
2	52.5	52.5	52.5	52.5	1965	0.1310	40.87	
3	77.5	77.5	79	78.3	2932	0.1151	20.49	
4	105	105	100	103.3	3868	0.1398	92.27	
				267.1	10000		224.89	

 $D = 0.602 \text{ cm.}^2/24 \text{ hours; } D_{20} = 0.606 \text{ cm.}^2/24 \text{ hours.}$

II.

Diffusion time: 2.840 days; temp. 16.8°–17.3°; average 17.0°.

	A	B	C	Average	0/000	x	$p \cdot x$	
1	25	27	29	27.0	1049	0.1540	81.47	$x_{av} = \frac{256.61}{1679} = 0.1528$
2	46.5	48	51.5	48.5	1885	0.1465	45.71	
3	73	80	73	75.3	2927	0.1142	20.33	
4	110	107	102.5	106.5	4139	0.1653	100.10	
				257.3	10000		256.61	

 $D = 0.547 \text{ cm.}^2/24 \text{ hours; } D_{20} = 0.569 \text{ cm.}^2/24 \text{ hours.}$

III.

Diffusion time: 3.042 days; temp. 19.5°–19.9°; average 19.7°.

	A	B	C	Average	0/000	x	$p \cdot x$	
1	48	46	46	46.7	1253	0.1327	70.20	
2	73	73	75	73.7	1978	0.1285	40.09	
3	108	107	112	109.0	2926	0.1141	20.31	$x_{av} = \frac{221.42}{1679} = 0.1319$
4	142.5	148	139	143.2	3843	0.1376	90.82	
				372.6	10000		221.42	

 $D = 0.592 \text{ cm.}^2/24 \text{ hours}$; $D_{20} = 0.594 \text{ cm.}^2/24 \text{ hours}$.*Carotene; first series.*

I.

Diffusion time: 2.930 days; temp. 19.3°–16.8° (mostly 17.8°–16.8°) average 17.3°.

	A	B	C	Average	0/000	x
1	39.5	40.5	—	40	851	0.1802
2	83	79.5	—	81	1724	0.1798
3	162	160.5	—	161	3426	—
4	193.5	182.5	—	188	4000	0.1518
				470	10001	

Section 1 + 2 = 2575; section 3 + 4 = 7426; $x = 0.1803$; $D = 0.450 \text{ cm.}^2/24 \text{ hours}$; $D_{20} = 0.467 \text{ cm.}^2/24 \text{ hours}$.

II.

Diffusion time: 2.920 days; temp. 18.2°–17.6°; average 17.9°.

	A	B	C	Average	0/000	x	$p \cdot x$	
1	—	8.6	9.0	8.8	962	0.1647	87.13	
2	—	14.2	13.9	14.05	1536	0.2228	69.51	$x_{av} = \frac{316.63}{1679} = 0.1886$
3	—	28.8	30.0	29.4	3215	0.2288	40.73	
4	—	38.4	40.0	39.2	4287	0.1807	119.26	
				91.45	10000		316.63	

 $D = 0.431 \text{ cm.}^2/24 \text{ hours}$; $D_{20} = 0.444 \text{ cm.}^2/24 \text{ hours}$.*Carotene; second series.*

I.

Diffusion time: 2.816 days; temp. 17.2°–16.5°; average 16.9°.

	A	B	C	Average	0/000	x	$p \cdot x$	
1	36	36	35.5	36	754	0.1935	102.36	
2	80	78	77.5	78.5	1644	0.1973	61.56	$x_{av} = \frac{327.69}{1679} = 0.1952$
3	148.5	147.5	148.5	148	3100	0.1614	28.73	
4	215.5	214	215	215	4502	0.2046	135.04	
				477.5	10000		327.69	

 $D = 0.432 \text{ cm.}^2/24 \text{ hours}$; $D_{20} = 0.450 \text{ cm.}^2/24 \text{ hours}$.

II.

	A	B	C	Average	0/000	x	$p \cdot x$	
1	28	27	27.5	27.5	872	0.1773	93.79	
2	53.5	53.5	53.5	53.5	1698	0.1854	57.84	$x_{av} = \frac{303.04}{1679} = 0.1805$
3	100	97.5	98.6	99.0	3143	0.1806	32.15	
4	135.5	136.5	132	135.0	4287	0.1807	119.26	
				315.0	10000		303.04	

 $D = 0.439 \text{ cm.}^2/24 \text{ hours}$; $D_{20} = 0.457 \text{ cm.}^2/24 \text{ hours}$.

Note. In Exp. 1 of the first series referring to carotene the concentration of section 3 was found very much too high and correspondingly the concentration of section 4 too low. Probably convection took place here.

In calculating the diffusion constant the sections 1 and 2 and 3 and 4 were added together and the value of x calculated by interpolating for the sum of these concentrations in the tables of Kawalki.

Discussion of the results.

The following table contains the summary of results referring to the diffusion constants:

Vitamin A.

	First series	Second series
I	$D_{30}=0.572$	$D_{30}=0.606$
II	$D_{30}=0.552$	$D_{30}=0.569$
III	$D_{30}=0.570$	$D_{30}=0.594$
Average	0.565	0.590
Average $D_{30}=0.578$ cm. ² /24 hours.		

Carotene.

	First series	Second series
I	$D_{30}=0.467$	$D_{30}=0.450$
II	$D_{30}=0.444$	$D_{30}=0.457$
Average	0.456	0.454
Average $D_{30}=0.455$ cm. ² /24 hours.		

The diffusion constant of vitamin A is found to be considerably higher than that of carotene, the difference exceeding appreciably the probable error of the experiments.

The results of parallel estimations of the vitamin experiments do not agree amongst each other as accurately as do other diffusion experiments done with the same apparatus. Probably the method of analysis must be considered as the principal source of inexactitude; as already stated the probable error of one determination amounts to 10 %. The accuracy of the observations was however enhanced by their number, considering that every value of the diffusion constant as recorded above is based on the average of 36 analyses. The accuracy of the analytical method for carotene exceeds that for vitamin A; this is perhaps the reason why the values found for carotene are more consistent than those for vitamin A.

Other sources of errors are the following.

1. Fluctuations of temperature; these were rather great for diffusion experiments. Xylene is not an advantageous solvent for these determinations. It has a small specific heat, a high dilatation coefficient and a low viscosity (drawbacks which however it shares with most other organic solvents). The occurrence of convection currents is very much favoured by these properties.

2. Evaporation of the xylene while filling and emptying the diffusion cylinders.

3. In the first series some glycerol was found at the bottom of the diffusion cylinder, after the experiments were finished.

4. Oxidation of the substance investigated.

Though the influence of these four sources of error upon the ultimate result is perhaps inferior to that involved by inadequacy of the analyses they doubtless vitiate the results to some extent. We estimate the probable error in the final figures above recorded (provided that there are no intrinsic causes due to systematic errors), for vitamin A 4 %, for carotene 2 %.

The molecular weight of vitamin A.

As to the molecular weight of vitamin A we may state the following. For the relation between the diffusion constant and the molecular weight, an empirical formula has been found:

$$D \sqrt{M} = c,$$

in which D is the diffusion constant, M the molecular weight and c a constant, dependent on the nature of the solvent and the temperature. This formula was experimentally tested by Öholm [1909] and found to be fairly satisfactory.

By comparing the diffusion constants found above with the molecular weights we obtain the following result:

$$\frac{\sqrt{M_{\text{car}}}}{\sqrt{M_{\text{vit}}}} = \frac{D_{\text{vit}}}{D_{\text{car}}} = \frac{0.578}{0.455} = 1.27.$$

If we now substitute in this formula the known value for the molecular weight of carotene we obtain:

$$M_{\text{vit}} = \frac{M_{\text{car}}}{1.27^2} = \frac{536}{1.61} = 333.$$

According to these calculations the molecular weight of vitamin A should be equal to 333, a figure which may be accepted only with reserve considering the uncertainty about the determination of the diffusion constant of vitamin A, and the error in M being twice as much as the error in D .

In addition we emphasise that the formula used above is not founded on the exact kinetic theory of diffusion; according to this the diffusion constant is connected more closely with the size, *i.e.* the diameter of the particles, than with their weight.

SUMMARY.

The diffusion constants of carotene and vitamin A in xylene were determined and found to be 0.455 cm.²/24 hours, and 0.578 cm.²/24 hours, respectively at 20°. The difference found exceeds considerably the probable errors of measurement. The ratio of the molecular weights of the two substances has been calculated and from this a molecular weight of about 330 has been deduced for vitamin A. The value obtained causes the assumption of a simple chemical relation between vitamin A and carotene to appear improbable.

REFERENCES.

- Bertram (1929). *Proc. Kon. Acad. Wetensch. Amsterdam*, **32**, 664.
Capper (1930). *Biochem. J.* **24**, 980.
Carr and Price (1926). *Biochem. J.* **20**, 497.
Cohen and Bruins (1923). *Z. physikal. Chem.* **103**, 349.
—— — (1924). *Z. physikal. Chem.* **113**, 157.
Euler, Euler and Karrer (1929). *Helv. Chem. Acta*, **12**, 278.
Kawalki (1894). *Ann. Phys. (Wied)*, **52**, 190.
Moore (1929). *Lancet*, ii, 380; *Biochem. J.* **23**, 803.
Öholm (1909). *Z. physikal. Chem.* **70**, 378.
Wolff, Overhoff and v. Eekelen (1930). *Deutsch. med. Woch.* **56**, 1428.

LIII. INTERMEDIARY CARBOHYDRATE METABOLISM.

THE EFFECT OF SODIUM IODOACETATE ON GLYOXALASE.

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INTRODUCTION.

THE keystone of a theory of intermediary carbohydrate metabolism, put forward by Dakin and Dudley [1913, 3; 1914] is the ketonic aldehyde, methylglyoxal. The postulation of this substance as the catabolic precursor and anabolic successor of lactic acid ($\text{CH}_3\text{.CO.CHO} \rightleftharpoons \text{CH}_3\text{.CHOH.COOH}$) correlates in an intelligible scheme many of the known metabolic changes of carbohydrates.

The discovery of the enzyme glyoxalase [Dakin and Dudley, 1913, 1], which, detectable in all mammalian tissues with the exception of the pancreas, rapidly converts methylglyoxal into lactic acid, provided substantial support for the theory, and a large body of evidence, subsequently collected, has upheld the opinion that methylglyoxal is, in fact, an intermediary compound in normal glycolysis [see *e.g.* Toenniesen and Fischer, 1926; Ariyama, 1928, 2].

Now Lundsgaard recently [1930, 1] independently rediscovered the fact, first noted by Pohl [1887], that the muscles of animals, poisoned with iodoacetic (or bromoacetic) acid, form no lactic acid under conditions in which normal muscle produces considerable amounts of this substance. Here then is a simple chemical substance which prevents the normal breakdown of carbohydrate, presumably by interfering with one or more of the catabolic reactions involved, and possibly, therefore, with that brought about by glyoxalase. The experimental examination of this possibility, which forms the basis of this paper, proves that sodium iodoacetate is in fact a powerful inhibitor of the action of glyoxalase.

EXPERIMENTAL.

In all the experiments here mentioned the breast-muscle of chickens was used as the source of the enzyme, since this tissue yields highly active extracts.

The technique of determining glyoxalase activity was that of Dakin and Dudley [1913, 4] in which the muscle extract is incubated with phenylglyoxal

in the presence of an excess of freshly precipitated calcium carbonate. *l*-Mandelic acid is produced, and a determination of its optical rotation gives a measure of the enzymic activity.

Glyoxalase is very sensitive to acids [Dakin and Dudley, 1913, 2] and it is for this reason that calcium carbonate is used to neutralise the acid formed during the reaction. It was obviously not permissible, therefore, in experiments on the effect of iodoacetic acid on the enzyme, to add the acid itself, and solutions of the neutral sodium salt were employed.

Numerous experiments concerning the effect of this salt on the action of glyoxalase *in vitro* were performed; in the interests of economy of space only one of these is described, which displays all the points of interest revealed in earlier experiments from which the best conditions for demonstrating the inhibitory effect of sodium iodoacetate were evolved.

Effect of sodium iodoacetate on the action of glyoxalase in vitro.

The minced breast-muscle of two chickens, which had been killed with chloroform, was digested with five times its weight of distilled water with frequent stirring at room-temperature for 2 hours. The extract was then filtered through muslin.

In the meantime a solution containing 3 mg. iodoacetic acid, neutralised with its equivalent of sodium hydroxide, per cc., a solution containing 20 mg. phenylglyoxal per cc., and an aqueous suspension of freshly precipitated calcium carbonate had been prepared.

All these liquids were first warmed to 37° and then in each of 8 flasks were placed 10 cc. of calcium carbonate suspension, 50 cc. of muscle extract and 2 drops of toluene. Each of the flasks 1-5 then received 10 cc. of sodium iodoacetate solution, and each of the flasks 6-8 (controls) 10 cc. of water. The flasks were kept at 37° and 10 cc. of phenylglyoxal solution were added to the series as follows: immediately to flasks 1 and 6; after 1 hr. to flask 2; after 2 hrs. to flasks 3 and 7; after 3 hrs. to flask 4; after 4 hrs. to flasks 5 and 8.

Each flask was withdrawn from the hot-room 20 hrs. after it received its phenylglyoxal, and the contents were worked up as follows.

25 g. ammonium sulphate were added and the flask was heated in a boiling water-bath for 5 mins. To the cooled contents were added cautiously 8 cc. of syrupy phosphoric acid. After standing for 30 mins. the liquid was filtered through a Büchner funnel; the precipitate was not washed. The filtrate was extracted 4 times with 10 cc. quantities of ether, and the combined extract was washed with 5 cc. water. The ether was then removed *in vacuo* and the residue dissolved in 7 cc. water. After filtration through paper in which a very small amount of charcoal had been placed the rotation of the Hg_{5461} line was read in a 1 dm. tube.

The experimental result is given in Fig. 1.

The points on curve *A* represent the rotations of the *l*-mandelic acid formed

in flasks 6, 7 and 8 and indicate that no appreciable loss of enzymic activity occurs when a glyoxalase solution is incubated in the presence of calcium carbonate at 37° for 4 hrs. before adding the substrate. The points on curve *B* represent the rotations of the *l*-mandelic acid formed in flasks 1–5 and the curve shows the rate of inactivation of glyoxalase by sodium iodoacetate under the prescribed experimental conditions.

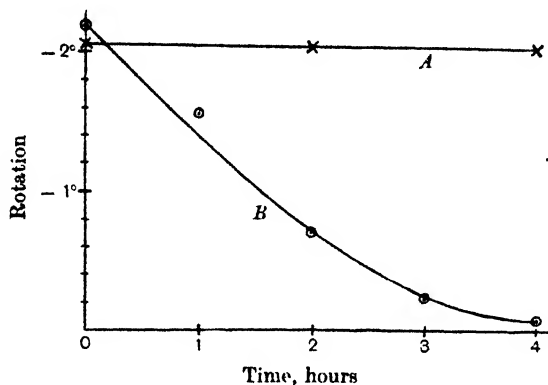


Fig. 1. *A* is the control glyoxalase curve. *B* shows the inactivation of glyoxalase by sodium iodoacetate.

To ensure comparable results in experiments of this kind the technique must be rigidly constant.

It is advisable to use flasks of equal capacity and shape. They should be large enough to allow the reaction mixture to lie in a shallow layer so that the calcium carbonate, which sinks to the bottom, may act reasonably efficiently as neutraliser of the acid formed. In these experiments 350 cc. Erlenmeyer flasks were employed.

The amount of toluene added as an antiseptic should be small and accurately measured since this substance itself has a definite inhibitory action on glyoxalase [Dakin and Dudley, 1913, 4].

The ether used for extracting the mandelic acid in experiments where iodoacetate has been added should be reasonably pure. Ether containing much peroxide liberates free iodine during the extraction. If this occurs a few drops of thiosulphate solution should be added to the water used for washing the combined ether extract.

Effect of sodium iodoacetate on glyoxalase in vivo.

4 cc. of a solution of sodium iodoacetate, equivalent to 26 mg. iodoacetic acid, were injected under the skin of the back into a chicken weighing 385 g. The first symptoms were noticed after 20 mins., the bird becoming lethargic. Its weakness steadily increased, until after $2\frac{3}{4}$ hrs. it died apparently from circulatory failure. A normal control bird of about the same weight was killed with chloroform and the breast-muscle of each was minced and digested for $2\frac{1}{2}$ hrs. at room temperature with five times its weight of distilled water. 50 cc. of each extract, filtered through muslin, were incubated at 37° for 16 hrs. with 10 cc. of calcium carbonate suspension and 10 cc. of a solution containing 0.2 g. phenylglyoxal, 2 drops of toluene being added to each flask as antiseptic. The mandelic acid formed was extracted in the manner already

described and the rotations were observed under the same conditions as in the previous experiment.

A second experiment in which a bird weighing 610 g. received 40 mg. iodoacetic acid as sodium salt in 4 cc. water was performed. The poisoned bird died $3\frac{1}{4}$ hrs. after the injection, and a control bird was killed for comparison at the same time. The rotations of the *l*-mandelic acid formed in the two experiments were as follows:

Normal chickens	Poisoned chickens
-2.06°	-1.24°
-2.02°	-1.19°

Although in many instances it is not justifiable to take tissues of one normal animal as controls against which to compare corresponding tissues of an experimental animal, it is the writer's experience that, with a rigid adherence to technique, the glyoxalase activity of a given tissue in a series of normal animals of the same kind is remarkably constant. The results quoted above are therefore considered to be significant, and the effect of poisoning with iodoacetic acid appears to have reduced the glyoxalase activity of the breast-muscle of these chickens nearly to one-half that of the same muscle of normal birds at a time when death supervenes.

DISCUSSION.

The experiments described in this communication demonstrate that sodium iodoacetate is a powerful inhibitor of the tissue enzyme which converts methylglyoxal into lactic acid.

The significance of this observation is considerably enhanced by Lundsgaard's investigations which have shown that sodium iodoacetate is not a general enzymic poison; it disturbs neither glycogenolysis nor the normal breakdown of phosphagen in a muscle in which it has completely suppressed the formation of lactic acid [Lundsgaard, 1930, 2]. In particular is it noteworthy that it does not interfere with the actions of the carbohydrate-splitting enzymes invertase and ptyalin, and, whilst it prevents the alcoholic fermentation of yeast, the oxidative metabolism is unaffected by it [Lundsgaard, 1930, 2, 3]. In the light of these interesting experiments on the scope of its action Lundsgaard forms the opinion that sodium iodoacetate inhibits specifically the process of glycolysis, interfering, therefore, at some stage of the degradation of glucose to lactic acid. The work here presented indicates a specific inhibition of the reaction which has been postulated as that immediately concerned with the production of lactic acid in tissues, thus supporting at the same time Lundsgaard's conclusion and the view that methylglyoxal is indeed the immediate precursor of lactic acid in the normal glycolytic chain of reactions.

Another inhibitor of the action of glyoxalase has been known for some time. In the course of their early work on glyoxalase Dakin and Dudley [1913, 4] observed that pancreatic extracts inhibited the action of the enzyme,

and they named the factor "antiglyoxalase." It is very interesting to note that both antiglyoxalase and sodium iodoacetate exert their effect on the enzyme in the same manner; neither inhibitor acts instantaneously, the degree of inhibition increasing with the length of time of contact between inhibitor and enzyme. The curve shown in Fig. 1 is of precisely the same type as is obtained in similar experiments with antiglyoxalase, and could be closely imitated by using an appropriate pancreatic extract.

Some time after Dakin and Dudley's description of antiglyoxalase Winfield and Hopkins [1915] announced that pancreatic extract was capable of inhibiting the formation of lactic acid in muscle.

The assumption of identity between antiglyoxalase and Winfield and Hopkins's factor was rendered difficult at that time mainly by their statement that the latter was heat-stable (and could therefore be neither trypsin, amylase nor lipase) whilst Dakin and Dudley had found that antiglyoxalase was readily destroyed by heat. Further differences appeared when Foster [1925] claimed to have separated them by means of 70 % alcohol in which the lactic acid-inhibiting factor was said to be soluble whilst antiglyoxalase was not. On this point, however, her evidence does not carry full conviction, for in several of her experiments the alcoholic extracts displayed antiglyoxalase action. It is true that this was slight in comparison with the inhibition of lactic acid formation in muscle caused by these preparations, but since she allowed her extracts to act on glyoxalase solutions for only 1 hr. before adding the substrate, it is clear, remembering that antiglyoxalase develops its inhibitory effect in a manner closely similar to that shown for iodoacetate (Fig. 1), that only slight inhibition would be expected, and that this would bear no simple quantitative relationship to the results which she obtained on the inhibition of lactic acid production in muscle.

Some confirmation of the inhibitory factor's heat-stability, as reported by Winfield and Hopkins, was perhaps provided by the partial survival of activity in her preparations after autoclaving for 20 mins. at 120°, although it should be noted that they were tested only with respect to antiglyoxalase.

When phenylglyoxal is incubated in muscle extracts which have been inactivated with respect to glyoxalase, or in pancreatic extracts which contain none of this enzyme, a yellow colour, or yellow precipitate, appears. Foster considered that this phenomenon most probably indicated that pancreatic extract acted on the substrate rather than on the enzyme, and doubted the existence of "antiglyoxalase" in the sense postulated by Dakin and Dudley. This conclusion has been negated by Ariyama [1928, 1], and recently Giršavičius [1930] has stated that the production of the yellow colour is entirely independent of the antiglyoxalase activity of the pancreas. Being apparently unaware of Dakin and Dudley's observation [1913, 1] that phenylglyoxal readily condenses with arginine, ornithine, histidine and lysine, yielding sparingly soluble yellow compounds, he rediscovered the clue to the proper explanation of the phenomenon, which is undoubtedly caused by

condensation of phenylglyoxal with basic amino-acids either in, or arising from, the protein of the tissue extracts.

Further investigations into the nature of the substance in pancreatic extracts which inhibits the formation of lactic acid by muscle have led McCullagh [1928] and Case and McCullagh [1928] to the conclusion that it is merely amylase, which acts by virtue of its power of preventing the formation of hexosephosphates, an explanation which has been challenged by Harrison and Mellanby [1930].

This conclusion is incompatible with Winfield and Hopkins's original observation on the heat-stability of the factor and with Foster's statement concerning its solubility in 70 % alcohol, for Harrison and Mellanby found that such solutions were practically devoid of amylolytic action.

As the problem stands at present, therefore, assuming the accuracy of the experimental observations briefly reviewed, it appears possible that there may be more than one mechanism whereby pancreatic extracts prevent the formation of lactic acid in muscle.

The significance and importance of antiglyoxalase in this connection is apparent from the work of Toenniessen and Fischer [1926; see also Ariyama, 1928, 2]. They have presented strong evidence that in a mixture of minced muscle and pancreas (or in mixed extracts of these organs) to which sodium fructosediphosphate had been added, methylglyoxal accumulated in detectable amounts. Similar experiments, using sodium iodoacetate as inhibitor, would be of considerable interest. According to Case and McCullagh's results, pancreatic amylase (or some associated substance) may interfere at an earlier stage of the degradation of carbohydrate; and the fact that a simple salt, sodium iodoacetate, inhibits lactic acid formation in muscle and the glyoxalase activity of muscle extracts, suggests the idea that there may occur in the pancreas a relatively simple compound, possibly heat-stable and soluble in 70 % alcohol, which also exerts an inhibitory power on glyoxalase, or on some other enzyme concerned in the process of normal glycolysis.

If this proved to be so, the conflict of evidence on the subject which has arisen in connection with this problem would be resolved.

In any event, however, there is good ground for the belief that the antiglyoxalase of pancreatic extracts does inhibit the formation of lactic acid in muscle by virtue of its inhibitory action on glyoxalase; and the fact that sodium iodoacetate, which suppresses the formation of lactic acid in muscle, also inhibits the action of glyoxalase, provides significant support to that belief.

SUMMARY.

1. Sodium iodoacetate, which Lundsgaard has shown to suppress lactic acid formation in muscle, is a powerful inhibitor of the enzyme glyoxalase which converts methylglyoxal into lactic acid. The available evidence supports the view that sodium iodoacetate owes its power of interfering with normal glycolysis to this fact.

2. The probability that methylglyoxal is the immediate precursor of lactic acid in the glycolytic chain of reactions is strengthened by this observation.

3. The inhibitory actions of sodium iodoacetate and pancreatic anti-glyoxalase on glyoxalase are similar in character: the significance of this finding is discussed in connection with the conflicting reports on the nature of the factor in pancreatic extracts which inhibits the formation of lactic acid in muscle.

REFERENCES.

- Ariyama (1928, 1). *J. Biol. Chem.* **77**, 359.
—— (1928, 2). *J. Biol. Chem.* **77**, 395.
Case and McCullagh (1928). *Biochem. J.* **22**, 1060.
Dakin and Dudley (1913, 1). *J. Biol. Chem.* **14**, 155.
—— (1913, 2). *J. Biol. Chem.* **14**, 423.
—— (1913, 3). *J. Biol. Chem.* **14**, 555.
—— (1913, 4). *J. Biol. Chem.* **15**, 463.
—— (1914). *17th Internat. Congr. Med.* London (1913).
Foster (1925). *Biochem. J.* **19**, 757.
Girsavičius (1930). *Biochem. J.* **24**, 446.
Harrison and Mellanby (1930). *Biochem. J.* **24**, 141.
Lundsgaard (1930, 1). *Biochem. Z.* **217**, 162.
—— (1930, 2). *Biochem. Z.* **220**, 1.
—— (1930, 3). *Biochem. Z.* **220**, 8.
McCullagh (1928). *Biochem. J.* **22**, 402.
Pohl (1887). *Arch. exp. Path. Pharm.* **24**, 149.
Toenniesen and Fischer (1926). *Z. physiol. Chem.* **161**, 254.
Winfield and Hopkins (1915). *J. Physiol.* **50**; *Proc.* v.

LIV. A FURTHER NOTE ON THE DETERMINATION OF IODINE IN BLOOD.

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IN a recent paper [Aitken, 1930] a method of estimating iodine in blood was described, the essential feature of which was that the iodine was concentrated into a very small volume of solution before being titrated. The titration was performed with a 0.1 cc. pipette, and gave satisfactory results provided the blood was not abnormally low in iodine. However, it is evident that in the case of a blood containing say only 40 γ of iodine per kg. the error involved in the use of a 0.1 cc. pipette and $N/1000$ thiosulphate will be at least 5 %. In order to eliminate such an error the author has devised a convenient and accurate micro-burette in which the standard solution floats on a thread of mercury. the position of the latter being adjusted by means of a fine screw projecting into a mercury reservoir.

The burette, shown in Fig. 1, was made from the stem of a discarded 200° thermometer which had a capillary of internal diameter about 0.18 mm. One end of this was drawn out to a tip *H* and bent over at *G* as shown. The other end of the capillary was opened out slightly and sealed into the steel chamber *A* by means of cement. *B* represents a "stuffing-box" (also of steel) containing vaselined packing which is used to ensure that the chamber *A* is mercury-tight. The "stuffing-box" is screwed on to the chamber *A* which is threaded at the lower end for this purpose, the pitch of the screw being about 0.8 mm. The chamber *A* contains mercury as shown by the black shading. The lower end of the "stuffing-box" (but not of the chamber *A*) carries an internal thread to accommodate the fine steel screw *D*, which is turned by means of the milled head *E* and serves to control the level of the mercury in the thermometer capillary. The pitch of this screw is 0.33 mm. and its diameter 1.4 mm., so that one complete turn of the milled head *E* expels about 0.0005 cc. of mercury from the chamber, corresponding to about 10 divisions on the stem. As regards the other dimensions of the various parts, they may

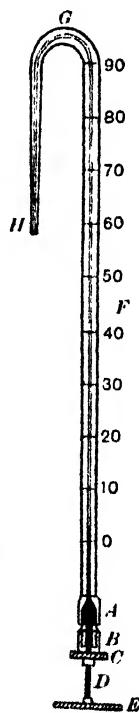


Fig. 1.

be gathered from the figure if it is remembered that in the actual apparatus the 10° graduations are 2 cm. apart.

The micro-burette, after being filled with mercury (which was done by filling the upper part of the "stuffing-box" with mercury and then attaching it to the chamber), was very carefully calibrated by drawing in threads of mercury at *H*, measuring their lengths in scale divisions and then weighing the threads. A table of corrections for variations in the diameter of the capillary was also drawn up, though these corrections were really negligible. The capacity of the burette showed that *N*/100 thiosulphate would be the most convenient standard to use, in which case 20 divisions on the scale corresponded to 0.21 γ of iodine in the sample.

The burette is filled with standard solution by immersing the tip *H* in a small vessel of the solution and rotating the milled head *E* forward until a small globule of mercury forms at the orifice; the head is then reversed and the standard solution drawn in to any convenient level. The tip is rinsed with distilled water, dried lightly with filter-paper and immersed in the solution in the titration-tube, and the titration is carried out by turning the milled head. Very delicate control is thus obtained as it is possible to raise the mercury level slowly through fractions of a scale division. In practice both the burette and the titration vessel are clamped to a burette stand, the whole being shaken bodily to ensure mixing during titration. The smallness of error arising in the burette can be gathered from the fact that it is easy to read to a quarter of a scale division on the stem and, using *N*/100 thiosulphate, this corresponds to 0.00262 γ of iodine in the sample.

Apart however, from inaccuracies in measurement, some investigators have found that a large error arises in estimations based on Fellenberg's method on account of the incompleteness of the alcoholic extraction of the potassium iodide. Thus Andrew [1930] finds it necessary to multiply his experimental results by 3/2, while Adolph and Shen-Chao Chen [1930] use the factor 100/85. Andrew [1930, p. 273] finds that there is little or no loss involved in the first alcoholic extraction, while there is a considerable loss in the second. This discrepancy, though perhaps partly due to losses by volatilisation, is probably mainly to be accounted for by differences in the conditions under which the extractions were performed. The present author has had no difficulty in obtaining a quantitative recovery of iodide, provided there is sufficient carbonate present to provide a paste which can be properly treated. In recent work he has found it convenient when performing the second alcoholic extraction to imitate exactly the conditions of the first alcoholic extraction. Small nickel crucibles (about 25 cc. capacity) are used instead of platinum dishes. To the first alcoholic extract in these crucibles is added about 0.4 cc. of the potassium carbonate solution and the residue after evaporation is incinerated, the small crucible being placed in a slightly larger one to avoid the risk of overheating. The residue is carefully moistened with carbonate solution so as to form a paste when rubbed with two small glass rods

flattened at the ends, and successive small amounts of alcohol are employed for the extraction. Thus the only difference between the extractions is one of scale, and it is possible to perform them with equal completeness.

The author wishes to express his indebtedness to Dr C. E. Hercus for the interest he has shown in this work.

REFERENCES.

- Aitken (1930). *Biochem. J.* **24**, 1456.
Andrew (1930). *Analyst*, **55**, 269.
Adolph and Shen-Chao Chen (1930). *Chinese J. Physiol.* **4**, 437.

LV. THE COLORIMETRIC DETERMINATION OF SODIUM.

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(Received February 23rd, 1931.)

No easy, rapid and accurate method of determining sodium in foodstuffs and other biological material is at present available. Such a method should preferably be applicable to the same hydrochloric acid extract of the incinerated material in which calcium, magnesium and potassium are being determined. Many of the published methods are gravimetric [Kramer and Tisdall, 1921] and therefore are not only slow, but require relatively large amounts. Doisy and Bell's [1920] caesium method admittedly presents considerable technical difficulties. Of the other methods examined by us the most promising appeared to be those depending on the precipitation of the sodium as the sodium uranyl zinc (or magnesium) acetate [Barber and Kolthoff, 1928; Caley and Foulk, 1929], and the subsequent estimation of the uranium in the precipitate, either by reduction and titration with potassium permanganate, or colorimetrically with potassium ferrocyanide. Barrensheer and Messiner [1927] have described a colorimetric method based on this principle suitable for serum or other biological fluids, but, as described by them, the method is very unsatisfactory. If, for example, the precipitate is washed according to their directions it goes completely into solution during the second washing. Poulsson [1928] modified the method to overcome this obvious defect, but the method is still not directly applicable to acid extracts of incinerated materials. We have therefore subjected the whole technique to a critical study, and have established a method which can be applied to neutral or acid solutions of sodium salts. A further communication will shortly be made on the application of the method to blood and serum without incineration.

Principle.

This depends on the removal of free acids and phosphates with zinc acetate and hydroxide in 50 % alcohol. The sodium is next precipitated as the triple acetate with uranium and zinc. The uranium in the precipitate is estimated colorimetrically with potassium ferrocyanide. The standard colour is obtained either by submitting a standard solution of sodium to the same treatment as the unknown, or by using a standard solution of the triple acetate.

Reagents.

(1) *Alcoholic zinc acetate with zinc hydroxide.* As all the samples of zinc acetate we have examined contained small amounts of sodium we prepare the acetate as follows. To a hot strong solution of A.R. zinc sulphate add a slight excess of ammonia (sp. gr. 0.880). Filter on a Büchner funnel, wash thoroughly with hot water and finally suck as dry as possible. To 12.5 cc. of glacial acetic acid add the zinc hydroxide paste, prepared as above, in small amounts at a time until in slight excess. Filter and wash; make up the combined filtrate and washings to 100 cc.; add 3.0 cc. of ammonia (sp. gr. 0.880) and 300 cc. of 95 % alcohol.

(2) *Alcoholic uranyl zinc acetate reagent* [modified from Kolthoff, 1927].
(a) Dissolve 10 g. of uranyl acetate in 50 cc. of boiling water containing 2.0 cc. of glacial acetic acid. (b) Dissolve 30 g. of zinc acetate in 50 cc. of boiling water containing 1 cc. of glacial acetic acid.

Mix both solutions while boiling, raise the temperature again just to boiling, allow to stand overnight and filter. Mix the filtrate with an equal volume of absolute alcohol, allow to stand 48 hours at 0° and filter at 0°. The reagent is stable at room temperature.

(3) *95 % alcohol saturated with the triple acetate.* Prepare a sample of sodium uranyl zinc acetate by adding the uranyl zinc acetate reagent to some NaCl dissolved in 50 % alcohol. Filter or centrifuge and wash the precipitate thoroughly with 95 % alcohol. Suspend the precipitate in 95 % alcohol and allow to settle in the ice-chest. Use the supernatant fluid for washing the precipitate. Filter before use if not absolutely clear.

(4) *20 % potassium ferrocyanide.*

(5) *Standard sodium chloride.* Dissolve 1 g. of pure dry NaCl in water and make up to 100 cc. This forms the stock solution. For use dilute 2 cc. to 100 cc. 1 cc. of this dilute standard contains 0.2 mg. NaCl (0.0786 mg. Na).

(6) *Standard triple acetate.* (a) *Preparation of the stock solution.* Take 10 cc. of a 1 % solution of NaCl, add 80 cc. of water and 100 cc. of alcohol. Add 100–120 cc. of zinc uranyl acetate reagent. Stand for at least an hour. Collect this precipitate quantitatively and wash it carefully with ice-cold 95 % alcohol. Dissolve the precipitate in water and make up to 1000 cc. This forms the strong stock solution. Dilute some of this accurately 1 to 5. Take 5 cc. of this weak solution, dilute with water in a 25 cc. flask. Add 1 drop of glacial acetic acid and 0.5 cc. 20 % potassium ferrocyanide. Make up to 25 cc. The resulting colour is close to that obtained from 0.2 mg. NaCl (0.0786 mg. Na) in 2 cc. of water submitted to all stages of the method and made up to 25 cc.

In the removal of phosphate it is necessary to add alcohol to the aqueous solution containing Na originally measured, and to take an aliquot portion of the mixture. Since there is a contraction on mixing alcohol and aqueous

solutions the exact value of this aliquot is not known and therefore this triple acetate solution must now be accurately standardised against Na solutions.

(b) *Standardisation of the solution.* This should be done at least in quadruplicate. Take four samples of exactly 0.2 mg. NaCl (0.0786 mg. Na) in 2 cc. of water and subject them to every stage of the estimation, including the steps for the removal of phosphates. Transfer the precipitates to 25 cc. flasks. In two other flasks take 5 cc. of the dilute standard and add water to about 18–20 cc. Develop the uranium colour as described below in all six flasks. Match both standards against each of the quadruplicate flasks, setting the latter at 20 mm. Take the mean (the individual readings should all agree within 3 %). Suppose this to be 23 mm. The simplest method of using the standard is now to set it always at 23 mm. in the colorimeter. This is equivalent to the colour obtained by estimation from 0.0786 mg. Na, the precipitate being made up to 25 cc. and the colorimeter set at 20 mm. and the results must be calculated on this basis (see below). Those who prefer it may make the necessary dilution of the stock solution so that 5 or 10 cc. of the weak standard diluted to 25 cc. gives exactly the same colour intensity as that obtained from 0.0786 mg. Na.

Detailed description of the procedure.

In a centrifuge tube take an amount of the unknown solution containing 0.04–0.16 mg. of sodium. (This may be described as the normal range of the method, but as shown below the method will estimate accurately and directly amounts up to 0.8 mg. Na.) Dilute to 2 cc. with water, add 4 cc. of the alcoholic zinc acetate reagent, stir and cover with a rubber cap (10 cc. vaccine caps are suitable). Allow to stand for 2–3 hours at room temperature and leave at 0° overnight. While still cold centrifuge and take 3 cc. of the supernatant liquid into another centrifuge tube. Add 4 cc. of the uranyl zinc acetate reagent and stir with a glass rod drawn out at its end to about 1 mm. thick. The stirring should be continued until the precipitate begins to appear. Cover with a rubber cap and allow to stand for one hour at 0°. Centrifuge, pour off the liquid and drain the tubes by inverting on filter-paper; wipe the mouths of the tubes, and wash once with 5 cc. of the ice-cold alcohol saturated with the precipitate, taking care that the whole of the inside of the tube is rinsed. The precipitate should be stirred up. Centrifuge, and drain again. Dissolve the precipitate in water and transfer it quantitatively to a 25 cc. volumetric flask. If the precipitate is very bulky, indicating that the unknown contained more than 0.15 mg. Na, transfer it to a 50, 100, or even 200 cc. flask according to discretion. After a little experience it is easy to judge the size of flask required from the bulk of the precipitate. For the standard either take 1 cc. of the dilute sodium chloride solution (containing 0.2 mg. NaCl per cc.) and treat it in exactly the same way as the unknown and transfer it to a 25 cc. flask; or, take 5 cc. of the dilute standard triple acetate solution in a 25 cc. flask. To both standard and unknown add 1 drop of glacial acetic acid and

0.5 cc. of 20 % potassium ferrocyanide, make up to the mark with water, allow to stand for 3 minutes and match. The amounts of acetic acid and potassium ferrocyanide are those required for 25 cc. flasks; if larger flasks are used, proportionately larger amounts must be taken.

Calculation.

(a) *Using a standard NaCl solution.*

$$\text{Na mg. per 100 cc.} = \frac{20 (\text{standard colorimeter reading})}{\text{Reading of unknown}} \times 0.0786 \times \frac{100}{\text{Volume of unknown taken}}.$$

(b) *Using the standardised triple acetate solution.* Suppose the colorimeter set at 23 mm. (see p. 451) is equivalent to 0.2 mg. of NaCl submitted to all stages of the estimation made up to 25 cc. and set in the colorimeter at 20 mm. Then, although the arbitrary standard was set at 23 mm., the calculation is:

$$\text{Na mg. per 100 cc.} = \frac{20}{\text{Unknown}} \times 0.0786 \times \frac{100}{\text{Vol. of unknown taken}}.$$

If 50 cc. or larger flasks have been used instead of 25 cc., the requisite additional factor must be introduced.

DISCUSSION.

1. *Effect of (a) Time, and*

(b) *Temperature on the precipitation.*

(a) Working with the same reagent, but in aqueous solution, Barber and Kolthoff [1928] found that precipitation was complete in $\frac{1}{2}$ hour and directed that the mixture be left for 1 hour. We have shown that with our reagent 1 hour is sufficient to give complete precipitation.

Table I. 0.0786 mg. Na taken for estimation.

Recovered at 0° (mg.)	
After 1 hour	After 18 hours
0.0785	0.0785
0.0782	0.0774
0.0793	0.0800
0.0762	0.0786

(b) We have found that from 0° to 10° the precipitation of sodium is complete, but that at 20° the precipitate is appreciably soluble and at 37°

Table II. 0.0786 mg. Na taken for estimation.

% recovered at			
0°	10°	20°	37°
100.0	100.5	92.7	79.6
100.5	98.0	95.0	81.6
99.5	99.2	92.7	80.3
100.0	100.5	92.1	84.6
Mean	100.0	99.8	81.5

much more so. The precipitation therefore should be carried out at 0° . Table II shows the extent of the error introduced.

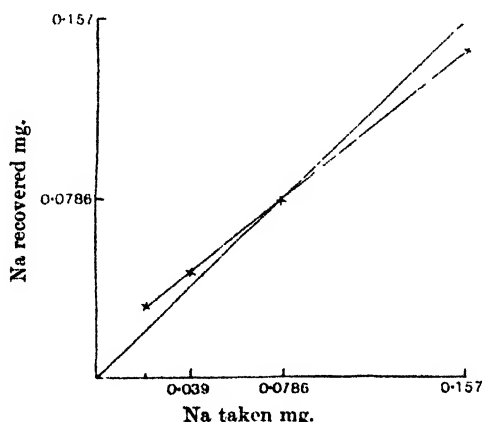
2. We have satisfied ourselves by direct experiment that the intensity of the colour developed by the potassium ferrocyanide is directly proportional to the amount of the triple acetate present. From a stock solution of the triple acetate 2 cc. of which contained about 0.039 mg. Na the following results were obtained: 1 cc., 2 cc. and 4 cc. were measured into 25 cc. flasks each in quadruplicate, 1 drop of glacial acetic acid and 0.5 cc. of potassium ferrocyanide were added to each and water added to make up to 25 cc. The flasks containing 2 cc. of stock triple acetate solution were taken as standard; they agreed with each other. The standard was then set at 20 mm. in the colorimeter and the other solutions matched against it. The results are shown in Table III.

Table III.

cc. triple acetate taken	Actual colorimeter reading mm.	Theoretical colorimeter reading mm.
1	39.4	40
1	39.4	40
1	41.0	40
1	40.5	40
4	10.0	10
4	10.1	10
4	10.0	10
4	10.0	10

The range of colour comparison is therefore correct for intensities from half to twice that of the standard.

3. When we first used this method we followed Kolthoff's [1927] directions for making up the precipitating reagent and did not add an equal volume of alcohol. On testing the method by taking known amounts of sodium we



Theoretical curve ———. Experimental curve x ——— x.
The colour developed from 0.0786 mg. Na is taken as standard and all the other values expressed as fractions of this.

Fig. 1.

invariably obtained the results shown in Fig. 1. Such a curve would have necessitated working with the colour of the unknown very close to that of the standard and would therefore have greatly restricted the range of the method.

This deviation from the theoretical recovery would be accounted for by the presence of 0.017 mg. of sodium as impurity in both the unknown and the standard. We had great difficulty in locating this impurity. Alcohol, distilled water and acetic acid were all suspected in turn, but the contaminating sodium was finally found in solution in the actual reagent used to precipitate the sodium. In the absence of alcohol some, or all, of the sodium present originally as an impurity in the zinc acetate remains in solution, and precipitates on adding the reagent to the alcoholic solution resulting from the removal of phosphates. After modifying the reagent the recoveries became theoretical (see Table IV below).

4. *Range of the method.* The amount of sodium precipitated by 4 cc. of reagent from the 2 cc. of aqueous solution taken is strictly proportional to the amount of sodium present from 0.02 to 0.8 mg. The larger amounts must be made up in larger flasks and the smallest made up to 8 cc. in the centrifuge tube without transference (see below under potassium interference).

Table IV.

mg. NaCl taken	Recovered	% recovery	mg. NaCl taken	Recovered	% recovery
0.05	0.0482	96.3	0.2	0.204	102
0.05	0.0502	100.5	0.2	0.200	100
0.05	0.0516	103.0	0.2	0.200	100
0.05	0.0500	100.0	0.2	0.202	101
0.1	0.097	97	0.4	0.405	101.3
0.1	0.100	100	0.4	0.405	101.3
0.1	0.100	100	0.4	0.416	104
0.1	0.098	98	0.4	0.412	103
0.2	0.192	96	2.0	2.05	102.5
0.2	0.195	97.5	2.0	1.94	97
0.2	0.195	97.5	2.0	2.04	102.0
0.2	0.206	103	2.0	1.96	98

Note. All the determinations were carried out against an arbitrary standard.

5. *The standard colour.* Although much easier to prepare, a solution of uranium acetate does not make such a satisfactory permanent standard as a solution of the triple acetate. This is because the zinc present in triple acetate solutions begins to precipitate as the colloidal ferrocyanide after some minutes. If the colour developed from triple acetate is compared in the colorimeter with that from a uranium acetate solution it will be found that the triple acetate colour remains stable for about 10 minutes and then appears slowly to become more intense. This increase is accompanied by the appearance of a colloidal precipitate which can only be seen when the solution is viewed by reflected light. The change, however, is slow, and we have used a uranium acetate standard quite satisfactorily by matching always between 3 and 8 minutes after developing the colour. All these difficulties are overcome by

using a solution of the triple acetate as standard. The colour comparisons are then stable for at least an hour, but we advise matching within 20 minutes of the development of the colour. The standardisation of this arbitrary standard has already been explained in detail under "Reagents."

6. *Interfering substances.* Caley and Foulk [1929] have shown that Ca, Mg, Sr, Ba and Fe do not interfere with this estimation of sodium. We can confirm their statement that Ca, Mg and Fe do not interfere. We have not tested Sr and Ba. The same authors report that lithium interferes with the precipitation, but there is practically no lithium in biological materials.

Phosphates are well known to be precipitated by uranium and must be removed. Table V shows that this is effectually accomplished by the zinc acetate in 50 % alcohol. A solution was prepared containing 9.82 mg. Na (25 mg. NaCl), 19.5 mg. K, 1.0 mg. Ca, 2.0 mg. Mg, 0.1 mg. Fe and 15.5 mg. P per 100 cc. The amount of sodium present in 1 cc. was estimated in quadruplicate. 1 cc. of a pure solution of NaCl containing 0.0786 mg. Na per cc. was also estimated in quadruplicate. All the colours were matched against an arbitrary triple acetate standard. The recoveries are given in Table V.

Table V.

Taken		Recovered	%
		mg.	
1 cc. pure NaCl - 0.20 mg. NaCl		0.201	100.5
" "	" "	0.203	101.5
" "	" "	0.199	99.5
" "	" "	0.199	99.5
1 cc. salt mixture = 0.25 mg. NaCl		0.248	99.3
" "	" "	0.243	97.4
" "	" "	0.248	99.3
" "	" "	0.251	100.5

Arsenates also are stated by Barber and Kolthoff [1929] to interfere, but we have not deemed it necessary to investigate their removal as they are absent from most biological material.

As potassium is often present in considerable excess over sodium in biological material, we have tested the effects of large amounts and have found that the method is satisfactory if the amount of potassium present in the volume taken for analysis does not exceed 0.6 mg. Table VI shows that interference is definite in the presence of 0.9 mg. K.

Table VI. 0.0786 mg. Na taken.

% recovered in the presence of	
0.62 mg. K	0.93 mg. K
97.0	120.5
100.7	119.2
100.0	119.2

0.6 mg. K is about 8 times the amount of Na used for the standard. In vegetable materials, e.g. grass, this ratio is often exceeded. When this is so the following modification of the method should be employed. Dilute the

specimen until 2 cc. contains less than 0.6 mg. K. Proceed as already described till the washing of the precipitate is completed. Now evaporate the residual drop of alcohol on a water-bath. Add 7.5 cc. of water and 0.5 cc. of 7 % potassium ferrocyanide acidified slightly with acetic acid. Match against the same standard that is always used. By this means it is possible to work with K/Na ratios of 32:1.

7. *Attention to detail.* Scrupulous care is essential when determining small amounts of Na. The element is ubiquitous so that the chances of contaminating the glassware and reagents are manifold. The authors recommend that the reagents be tested for purity from time to time in the following simple way. Take 0.04 and 0.08 mg. Na (or 0.1 and 0.2 mg. NaCl), make each up to 2 cc. and submit these to every stage of the sodium estimation. Make up the precipitates to 25 cc. Develop the colours and set the stronger at 20. The weaker should read 40. If it reads less than 40 one or more of the reagents contains sodium.

SUMMARY.

1. A method is described by which 0.02–0.8 mg. Na may be directly determined. In the absence of phosphates the range is 0.01–0.4 mg. Na.

2. The sodium is precipitated as sodium uranyl zinc acetate and the uranium is determined colorimetrically with potassium ferrocyanide.

3. The method is applicable to neutral or acid solutions.

4. Ca, Mg and Fe do not interfere. Phosphates interfere, but are removed with zinc acetate.

5. Sodium may be determined directly in the presence of 30 times its weight of potassium.

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REFERENCES.

- Barber and Kolthoff (1928). *J. Amer. Chem. Soc.* **50**, 1625.
—— (1929). *J. Amer. Chem. Soc.* **51**, 3233.
Barrensheer and Messiner (1927). *Biochem. Z.* **189**, 308.
Caley and Foulk (1929). *J. Amer. Chem. Soc.* **51**, 1664.
Doisy and Bell (1920). *J. Biol. Chem.* **45**, 313.
Kolthoff (1927). *Z. anal. Chem.* **70**, 397.
Kramer and Tisdall (1921). *J. Biol. Chem.* **46**, 467.
Poulsen (1928). *Biochem. Z.* **193**, 423.

LVI. STUDIES ON THE METABOLISM OF ANIMALS ON A CARBOHYDRATE-FREE DIET.

II. VARIATIONS IN THE SENSITIVITY TOWARDS INSULIN OF DIFFERENT SPECIES OF ANIMALS ON CARBOHYDRATE-FREE DIETS.

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IN each set of experiments described in our previous communication [Hynd and Rotter, 1930], a number of animals were tested for their response to insulin. It was thus possible to compare the effects produced by an injection of the hormone not only with regard to the previous dietetic treatment of the animal, but also in relation to the existing blood-sugar level, the muscle-glycogen content, and the distribution of glycogen and fat in the liver.

All the experiments were conducted in a room the temperature of which was maintained at 20-22°, for although the symptoms develop less rapidly at this temperature than at higher temperatures such as 30-37°, as first shown by Krogh, our conditions are more normal and hence less likely to bring other factors into operation. The same sample of insulin was used throughout the experiments recorded in this paper, and the injections were made subcutaneously. The dose employed for the several species varied slightly, the amount used being that found necessary to produce in control animals the train of symptoms accompanied by what are usually referred to as "hypoglycaemic convulsions."

Experiments with mice.

For the experiments with mice the insulin was diluted 1 in 10 with 0.05 % hydrochloric acid, 7 minims of this diluted insulin being the dose for a mouse of 20 g. body weight. As the diets employed have already been described [Hynd and Rotter, 1930], it is only necessary to indicate the number of animals employed in the several experiments. On carbohydrate-free diets 41 mice were tested for their response to insulin, namely 15 on cheese, 14 on butter-fat, and 12 on caseinogen, and these tests were controlled by 23 animals fed on a normal diet.

With one exception all the mice on the control diet showed the characteristic insulin symptoms between 1 and 1½ hours after the injection, and re-

quired to be resuscitated by the administration of glucose or of maltose. The cheese-fed mice, on the other hand, were without exception resistant to the same dose of insulin, and, even with twice the dose, the only effect of the injection of insulin was to render the animals somewhat quiet and drowsy. All returned to normal in 2-3 hours without treatment of any kind. To elicit severe symptoms including convulsions, the dose required to be increased 3-4 times. Cheese-fed mice, which had been proved resistant to insulin, showed a normal response after 12 days' subsequent feeding on oats, marked lowering of the blood-sugar (0.062) occurring in 30 minutes after the insulin injection and the convulsive level (0.054) being reached in 65 minutes.

Similar results were obtained with mice reared on caseinogen until 7 weeks old, although one animal died 30 minutes after being injected. Of the remaining 11 mice used, 7 showed no symptoms except drowsiness, and the other 4, in addition to being drowsy, showed slight sprawling of the limbs. This, however, did not develop further and the animals were normal in the course of 3 hours. Mice rendered resistant to insulin by a diet of caseinogen remained so, though supplied for 5 days with as much (*a*) glucose, or (*b*) fructose, as they would take in addition to the usual amount of caseinogen. These experiments were not continued further, as the animals refused after a time to eat the pure sugars.

Of the 14 fat-fed mice, 6 showed no symptoms except drowsiness, 6 others became slightly sprawling for a time, and only 2 showed severe symptoms requiring glucose injection to bring about recovery.

Evidently either for maintaining the growth and health of the animals, or for producing resistance to the effects of insulin, a carbohydrate-free diet, consisting exclusively of either fat or protein, is not so satisfactory as one in which both protein and fat are present. Nevertheless, on all the carbohydrate-free diets employed, mice showed a marked difference in their sensitivity towards insulin from animals on a normal diet. After 2 weeks' feeding on a carbohydrate-free diet, resistance to insulin is well established, and the mice were still resistant when the cheese diet had been continued for 2½ months.

Experiments with rats.

From the experience gained in feeding carbohydrate-free diets to mice, only two diets were attempted with rats, namely a carbohydrate-rich diet of bread and milk, and a carbohydrate-free diet of cheese. Twenty-two animals on each of these diets have been tested with insulin, the dose being proportionately less than that given to mice, namely 10 minims of 1 in 5 insulin per 100 g. of body weight. As we found that the rate at which hypoglycaemic symptoms developed in a normal rat seemed to depend, in part at least, on the strength of the insulin solution injected, we have used invariably for the experiments with rats 1 part of insulin diluted with 4 parts of 0.05 % HCl. Also as piebald rats are somewhat variable in their response to insulin, all the experiments here reported were carried out on albinos.

Under the dosage indicated above, rats on the cheese diet showed no notable symptoms except drowsiness, while the control rats without exception had severe convulsions after about 2 hours, and required to be relieved by the injection of glucose.

The following is typical of the different behaviour of the two sets of rats.

	Rat, ♂, 161 g. Diet of bread and milk	Rat, ♂, 160 g. Diet of cheese for 14 days
9.43 a.m.	Injected with 16 minims of 1:5 insulin	Injected with 16 minims of 1:5 insulin
11.0 ..	Not quite normal; climbs wire cage on being disturbed	Not quite normal; remains quiet
11.5 ..	Irritable; no sprawling	Legs sprawling; feels rigid when handled; no fall of body temperature; not irritable
11.15 ..	Symptoms developing	Improving; looks drowsy; otherwise almost normal
11.45 ..	Sprawling limbs; cold and limp	Quiet and drowsy; otherwise normal
11.51 ..	Severe convulsion	Drowsy
11.57 ..	Severe convulsions	Drowsy
12.1 p.m.	Severe convulsion	Drowsy
12.5 ..	Another convulsion	No further symptoms developed
12.10 ..	Injected 1 cc. 20% glucose solution	Taking food (cheese moistened with milk)

In the experiments with rats, it has been noticed that almost invariably, previous to the stage when symptoms develop in the carbohydrate-fed animals, the cheese-fed rats appear somewhat worse than the controls. They are not so irritable, but much less active, and show slight sprawling of the hind limbs. However, they do not become cold and when handled appear rigid rather than limp. This effect lasts only a very short time, and the animals then improve without further symptoms developing. If the dose of insulin has been too large, or the carbohydrate-free diet has not been continued long enough, mild insulin symptoms may develop, but if so, they appear later than in the control rats, as noted by Bainbridge [1925].

As we have already shown that the livers of cheese-fed rats have a maximum fat content after a fortnight's feeding, experiments were carried out to determine (1) whether the resistance to insulin is also at a maximum at the same time, and (2) when resistance first develops. The results obtained were inconclusive. Partial resistance to insulin could be detected after 4 days on the cheese diet, as after this period of feeding the symptoms were delayed in development and less severe than those of the control animals. After 8-10 days, resistance was well established, and was still well marked after 1 month. This is in agreement with the observations of Abderhalden and Wertheimer [1924, 1], and of Bainbridge [1925]. The former workers noted resistance in rats after 10 days' feeding, while the latter employed periods of 2-6 weeks' feeding. In our experiments, all animals after 14 days' feeding on cheese were resistant to insulin. It was also found that, after the withdrawal of food for 24 hours, rats which had been fed on cheese for a fortnight were still resistant to the effects of insulin.

Experiments with rabbits.

Only one experiment was carried out on rabbits, in which we confirmed the observation made by several workers (Page [1923], Blatherwick *et al.* [1924], Abderhalden and Wertheimer [1924, 3], Pénaud and Simonnet [1925], Geiger and Kropf [1929]), that rabbits on green food are less resistant to insulin than animals fed on oats. The object of our experiment, however, was not merely to confirm this behaviour of rabbits, but rather to determine whether any departure from the normal could be detected in the respective reducing power of the blood-plasma and corpuscles of such animals. As is evident from the results tabulated below, no significant differences were obtained on the two types of diet.

Diet	Whole blood-sugar %	Plasma- sugar %	Corpuscle- sugar %	Plasma volume	:	Corpuscle volume
Greens	0.113	0.144	0.047	76.66	:	23.34
Oats	0.134	0.147	0.060	77.71	:	22.29

Experiments with kittens.

As we had found previously that kittens were more responsive than rabbits to the same dose of insulin, it seemed desirable to extend our observations to this species. The carbohydrate diet was again bread and milk, while the carbohydrate-free diets employed were (*a*) white fish, and (*b*) cream, the former consisting largely of protein, the latter having a high fat content, and both suited to the type of animal under investigation. Four sets of experiments have been carried out. In each set the kittens employed were of the same litter and were 6 weeks old at the commencement of the experiment. After 3-4 weeks on the diets indicated above, they were injected subcutaneously with 20 minims of insulin per kg. of body weight.

The kittens on all three diets responded to the injection and displayed the characteristic symptoms, including leg-weakness, mewing, salivation and convulsions [Olmsted and Logan, 1923]. Relief followed the administration of glucose, but there were frequent relapses. Hence a smaller dose of insulin, namely 10 minims per kg., was tried, but with similar results. Typical symptoms ensued in all cases, and the only difference noted was the length of time required for the symptoms to develop. The time of onset of the first convulsion in each case is summarised in the following table, from which it is seen that the animals on the carbohydrate-free diets were always much earlier in showing symptoms than the carbohydrate-fed animals. In fact, on two occasions the kittens fed on bread and milk did not have convulsions.

Period which elapsed before first convulsion (minutes)

Diet	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Fish	60	75	75	75
Cream	80	80	90	—
Bread	180	(No convulsion)	(No convulsion)	105
Minims of insulin	20	10	10	10

DISCUSSION.

On a carbohydrate-free diet rats and mice, while showing no significant change in muscle-glycogen when compared with animals on a carbohydrate-rich diet, tend to have a high blood-sugar, an increased liver-fat content, and a diminished liver-glycogen [Hynd and Rotter, 1930]. Can any of these differences be correlated with the resistance to insulin that such animals develop when maintained on a carbohydrate-free diet?

In the table which follows are summarised the blood-sugar levels of mice on different diets and their conditions at varying periods after the injection of insulin. On the control diet of bread and milk, the blood-sugar is seen to fall rapidly and the convulsive level is soon reached. On the carbohydrate-free diets, on the other hand, though the actual range through which the blood-sugar is lowered by insulin is approximately the same, the change takes

Experiments with mice.

Time after insulin injection (mins.)	Diet ...	Without being fasted						Fasted 18 hours			
		Bread		Cheese		Fat		Bread		Cheese	
		Blood-sugar %	Con- dition of animal	Blood-sugar %	Con- dition of animal	Blood-sugar %	Con- dition of animal	Blood-sugar %	Con- dition of animal	Blood-sugar %	Con- dition of animal
0	0.124	Normal		0.157	Normal	0.191	Normal	0.174	Normal	0.171	Normal
30-40	0.05	Very irritable		0.087	"	0.076	"	0.087	Slightly affected	0.073	"
50-60	0.039	Convulsion		—	"	0.039	"	0.056	Convulsion	—	"
70-80	0.038	"		0.040	"	—	"	—	"	—	—
90	0.039	"		—	"	0.049	"	—	—	—	—
150	—	—		0.057	"	0.056	"	—	—	0.064	Slightly sprawling
240	—	—		0.131	"	—	"	—	—	0.064	Normal
Max. fall	0.086	—		0.100	—	0.142	—	0.118	—	0.107	—

place much more slowly and the animals remain more or less normal. Further, as animals which have fasted 12-24 hours have their blood-sugars at the same level as controls, but are still resistant to insulin, it is not the initial high blood-sugar level but rather the diminution in the rate at which the fall in blood-sugar is produced that is concerned with the resistance to insulin. This is supported by the experiments with rats on carbohydrate-free diets carried out by Abderhalden and Wertheimer [1924, 2], who have reported that such animals after the injection of insulin display a less rapid fall in blood-sugar. Tiitso [1925] also found that the blood-sugar falls less rapidly in starved rabbits than in those fed with abundant carbohydrate.

Since the blood-sugar level represents the balance between two sets of opposing processes, it is necessary to inquire if we can discover any factor responsible for the diminished rate at which the blood-sugar falls in non-carbohydrate-fed rodents, since it appears that there is some connection between the diminution in rate of fall of blood-sugar and the response to insulin.

The difference in the effects of insulin on well-fed as compared with starved animals was considered by McCormick, Macleod, Noble and O'Brien [1923] to depend primarily upon the amount of liver-glycogen. Lawrence [1926], also, in emphasising the importance of the glycogen content, stated that when the glycogen stores were empty insulin was much more effective in its hypoglycaemic action than at any other time. Accordingly, as we have found that animals on a carbohydrate-free diet show a reduced liver-glycogen content, one would predict that such animals should respond more readily to an injection of insulin. Experiment, however, shows just the opposite result. Non-carbohydrate-fed rodents withstand insulin much better than those receiving abundant carbohydrate, both when judged by the rate of fall of the blood-sugar and the incidence of convulsions. The amount of glycogen stored in the liver, therefore, is not, in all circumstances, a reliable index of the sensitivity of an animal towards insulin. In other words, there is some factor more important than the available amount of stored glycogen.

Bainbridge [1925] believed resistance to be due to excess of fat rather than to absence of carbohydrate from the diet, and sought to establish a connection between resistance to insulin and the fat content of the diet. Our results do not support this contention. The livers of rats and of mice on a cheese diet show a high liver-fat content, which reaches a maximum at about that period when maximum resistance to insulin develops, but as the excess of liver-fat is extremely mobile and disappears after 24 hours' starvation, lack of response to insulin is not connected with the excessive amount of fat, for starved animals are still resistant to insulin. Moreover, we have succeeded in producing marked resistance by feeding mice on a purely protein diet, namely on caseinogen, while several workers have reported on the resistance of rabbits fed on diets containing little or no fat. Again, it is well recognised that animals on a high carbohydrate diet after a time become distinctly fatty. Yet such fattened animals do not show resistance to insulin. On the other hand, the absence of carbohydrate from the diet was considered by Abderhalden and Wertheimer [1924, 1, 2] to be a factor of importance in establishing resistance to insulin. Some support for this opinion is found if one accepts Laufberger's [1924] view that the essence of insulin action is to inhibit gluconeogenesis, for when no preformed carbohydrate is supplied, the formation of carbohydrate from other substances must of necessity become accentuated, and consequently a diminished insulin effect would result in animals on a carbohydrate-free diet. However, the investigations of Grevenstuk, de Jongh and Laqueur [1925] indicated that the resistance was not due to any specific type of foodstuff, but depended on the relative proportions of carbohydrate, protein and fat.

Generally speaking our observations on the resistance of rodents to insulin are in agreement with those recorded by other workers: and it is quite clear that neither the stores of glycogen nor of fat are responsible for the resistance to insulin. But a further difficulty arises when the results of the experiments

with kittens are considered, because in this type of animal neither a diet consisting entirely of protein, nor one containing a large amount of fat, brought about resistance to insulin. In fact, our experiments showed that kittens on a carbohydrate-rich diet were somewhat less responsive to insulin than animals on carbohydrate-free diets. However, the carbohydrate-fed kittens could not be described as resistant to insulin, as, though in two cases no convulsions occurred, the animals were definitely affected. Had a smaller dose of insulin been given, resistance might have been claimed, but the chief point for the present is that kittens differ from mice and rats in that feeding a carbohydrate-free diet does not render them resistant to the action of insulin.

Eadie [1930] has recently reported a difference in the action of adrenaline in the cat and the rat, and Corkill [1930] found the young rabbit to differ from several other species with regard to the increase of liver-glycogen after insulin. Funk [1922], also, has pointed out that dietary changes have often an influence on the toxicity of certain drugs. Consequently we have here a parallel to our results with regard to the action of insulin. In other words, there is a distinct species difference not only between kittens, which may be taken as representative of carnivora, and rodents, but also amongst the rodents themselves.

Now it is well known that while the alkali reserve of a rabbit is easily changed, it is exceedingly difficult to alter that of a dog or a man, and hence probably also that of a cat [McClendon, 1919]. Feeding on oats was found by Page [1923] and also by Abderhalden and Wertheimer [1924, 3] to lower the alkali reserve of a rabbit, and a non-carbohydrate diet would be capable of a similar effect in the case of a mouse or a rat [Abderhalden and Wertheimer, 1925], but not in a dog, cat or man [Michalowsky, 1930]. We thus arrive at the reasonable conclusion that an animal when maintained on a diet which is capable of bringing about a lowering of its alkali reserve will tend to show resistance to the action of insulin. There is thus an element of truth in the views of both Abderhalden and Wertheimer [1924, 1, 2] and of Bainbridge [1925], already referred to, as either absence of carbohydrate or excess of fat inevitably leads to a diet tending to reduce the alkali reserve. Though certainly not the only factor, the acid-base equilibrium of the body is thus a fundamentally important one in the mechanism of insulin action, in that it probably regulates the effect not only of the internal secretion of the pancreas, but also the effects of the other ductless glands, such as the suprarenals, thyroid and pituitary, which are known to be concerned in carbohydrate metabolism. In a forthcoming paper further experiments will be described in support of this view.

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REFERENCES.

- Abderhalden and Wertheimer (1924, 1). *Pflüger's Arch.* **203**, 439.
—— — (1924, 2). *Pflüger's Arch.* **205**, 547.
—— — (1924, 3). *Pflüger's Arch.* **205**, 559.
—— — (1925). *Pflüger's Arch.* **207**, 222.
Bainbridge (1925). *J. Physiol.* **60**, 293.
Blatherwick, Long, Bell, Maxwell and Hill (1924). *Amer. J. Physiol.* **69**, 155.
Corkill (1930). *Biochem. J.* **24**, 779.
Eadie (1930). *Amer. J. Physiol.* **94**, 69.
Funk (1922). *The Vitamines*, 2nd ed., trans. by Dubin.
Geiger and Kropf (1929). *Arch. exp. Path. Pharm.* **139**, 290.
Grevenstuk, de Jongh and Laqueur (1925). *Biochem. Z.* **163**, 357.
Hynd and Rotter (1930). *Biochem. J.* **24**, 1390.
Laufberger (1924). *Klin. Woch.* **3**, 264.
Lawrence (1926). *Quart. J. Med.* **20**, 69.
McClendon (1919). *J. Biol. Chem.* **38**, 539.
McCormick, Macleod, Noble and O'Brien (1923). *J. Physiol.* **57**, 234.
Michalowsky (1930). *Klin. Woch.* **9**, 1505.
Olmsted and Logan (1923). *Amer. J. Physiol.* **66**, 437.
Page (1923). *Amer. J. Physiol.* **66**, 1.
Pénau and Simonnet (1925). *Compt. Rend. Soc. Biol.* **93**, 1292.
Tiiitso (1925). *Proc. Soc. Exp. Biol. Med.* **23**, 40.

LVII. EFFECT OF THE ADDITION OF SALTS ON THE p_H OF SOLUTIONS OF AMPHOLYTES.

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It has been found that the velocity of hydrolysis of starch in the system gelatin-starch-amylase is affected by the addition of calcium chloride. This effect may be due to change in the hydrogen ion concentration of the system.

Such a possibility was first pointed out by Spiro [1922], who found that the p_H of glycine solutions was lowered by addition of calcium or potassium chloride, and similar results were obtained by Mond [1923], Netter [1925], Simms [1929], and others. The majority of these authors interpreted the result of addition of electrolytes to colloidal sols as being essentially the same as in the case of crystalloid ampholytes, such as glycine.

Such a view is not, in our opinion, sufficiently justified by the experimental material available, as the effects of relatively high concentrations (0.15–0.25 M) of electrolyte only were examined, and, further, the dependence of the observed phenomena on the concentration of electrolyte was not investigated. The work of Freundlich [1930] on hydrophobe, and of Kruyt [1928, 1929] on hydrophile, colloids has shown that the influence of salts on the electrolytic properties of colloidal particles should rather be evidenced by low (0.02 M) than by high concentrations of salt.

In the present paper, experiments on the change in p_H of solutions of gelatin, ovalbumin, peptone, and glycine will be described, in relation to the concentration within the limits 0–0.025 M of salt added, to the valency of the anion and cation of the salt and to the original p_H of the solution.

EXPERIMENTAL.

Gelatin (Coignet et Cie, Paris) was twice washed with cold distilled water, and dissolved at 60°. 100 cc. portions of 0.5–2.0 % solutions were placed in flasks containing 10 cc. of water or of various salt solutions, and the p_H of the systems was determined potentiometrically at 20°, using a hydrogen-calomel electrode. Special attention was paid to the purity of the water and to the cleanliness of the vessels used, since even traces of impurity might affect the colloidal state of the gelatin. The salts used were sodium, potassium,

calcium, and magnesium chlorides and sodium and magnesium sulphates (Merck's "chemisch rein" chemicals). The p_H of the salt solutions was brought to that of the gelatin solution by the addition of 0.02–0.1 *N* sodium hydroxide, which was also used for bringing the gelatin solution to the desired p_H .

Similar experiments were performed using Witte's peptone (Poulenc Fr.); this peptone contained colloidal matter precipitable by the addition of 50 % alcohol. The ovalbumin used (Merck) contained globulin, which, however, separated on standing and was removed by filtration.

I. Gelatin.

These experiments (Table I) were performed on 1 % solutions of gelatin, the p_H of which was 5.8–5.9. It appears that whilst the salts used act quantitatively differently, yet in all cases the p_H of the system is lowered. This is best illustrated by Table I, which shows that the depression of p_H is, except at very low concentrations, proportional to the concentration of potassium chloride. Bivalent anions and cations act differently, being much more active per unit of concentration when present in small (1–10 *mM*) than in higher concentrations, and the curves obtained for such salts are of the type of adsorption isotherms.

Table I. *Variations in p_H of gelatin solutions.*

Molar concentration of salt	Lowering of p_H				
	CaCl ₂	MgCl ₂	MgSO ₄	Na ₂ SO ₄	KCl
0.0025	0.21	0.12	0.09	0.06	—
0.005	0.25	0.20	—	—	—
0.010	0.30	0.26	0.19	0.15	—
0.020	0.39	0.33	—	—	—
0.025	0.43	0.38	0.27	0.26	—
0.050	—	—	—	—	0.09
0.100	—	—	—	—	0.17

The results obtained in systems in which the concentration of calcium chloride is maintained constant whilst that of gelatin is varied (0.4–1.2 %) are given in Table II, in which we see that the depression in p_H caused by the salt is greater at lower than at higher concentrations of gelatin.

Table II. *Influence of concentration of gelatin.*

Gelatin %	Concentration of CaCl ₂ <i>M</i>	Lowering of p_H
0.4	0.010	0.38
	0.020	0.59
	0.010	0.48
0.6	0.020	0.50
	0.010	0.44
0.8	0.020	0.46
	0.010	0.44
0.9	0.020	0.44

The reaction of gelatin solutions was varied by adding 0.02 *N* sodium hydroxide solutions to the required p_H ; in this way three solutions were prepared, of p_H 5.90, 8.03, and 9.21, from the same preparation of gelatin, and of the same concentration. The greatest depression of p_H due to the addition of electrolytes was found at p_H 8.03 (Table III), 0.005 *M* calcium chloride lowering the p_H by 0.46, whilst at p_H 5.90 (Table I) the observed depression is only 0.25, and at p_H 9.21 0.03; the corresponding depressions observed in the presence of 0.01 *M* salt are respectively 0.54, 0.30 and 0.05. The solution remained clear at p_H 5.90 and 8.03, whilst at p_H 9.21 opalescence was observed on the addition of calcium chloride. Substantially similar results were obtained with magnesium chloride in place of calcium chloride.

Table III. *Influence of original p_H .*

Concentration of CaCl_2 <i>M</i>	p_H	Lowering of p_H
0.0	8.03	0.0
0.005	7.57	0.46
0.010	7.49	0.54
0.0	7.44	0.0
0.0025	7.23	0.21
0.0	9.21	0.0
0.005	9.18	0.03
0.010	9.16	0.05
0.025	9.01	0.25

II. Ovalbumin.

These experiments were performed on 2 % aqueous solutions of ovalbumin which were filtered overnight through filter-paper. The effect of adding calcium chloride or magnesium sulphate to such solutions is given in Table IV; the curve connecting variation in p_H with concentration of electrolyte is also of the adsorption isotherm type.

Table IV. *Ovalbumin solutions.*

Salt concentration <i>M</i>	p_H	Depression of p_H
CaCl_2 0.0	8.58	0.0
„ 0.005	8.42	0.16
„ 0.010	8.37	0.21
„ 0.020	8.32	0.26
„ 0.025	8.31	0.27
MgSO_4 0.010	8.47	0.11

III. Peptone.

The action of various concentrations of calcium and magnesium chlorides, magnesium and sodium sulphates, and sodium chloride on 3 % peptone solutions at p_H 4.8 and 5.7–6.0, was examined. At the isoelectric point for peptone (p_H 4.8) it was found that electrolytes possessing a bivalent anion (sodium sulphate) or cation (magnesium or calcium chloride), or both together (magnesium sulphate) produced only a negligible depression of p_H (Table V).

Table V. *Peptone.*

Concentration <i>M</i>	Depression of p_H			
	CaCl_2	MgCl_2	MgSO_4	Na_2SO_4
Original $p_H = 4.78-4.80$:				
0.005	0.05	0.06	0.05	0.05
0.010	0.07	0.09	0.07	0.07
0.025	0.08	0.12	0.09	—
Original $p_H = 5.8-6.0$:				
0.010	0.23	—	0.17	—
0.025	0.42	0.38	0.38	—

Solutions brought to p_H 5.7-6.0 by the addition of sodium hydroxide exhibit, on the other hand, a marked depression of p_H on the addition of bivalent ions. The magnitude of the change in p_H is a function of the initial p_H of the solution.

IV. *Glycine.*

The results obtained (Table VI) are identical with those obtained by earlier workers.

Table VI. *Glycine.*

Concentration of CaCl_2 <i>M</i>	p_H of solution	Lowering of p_H
0	6.78	—
0.025	6.71	0.05
0.050	6.66	0.12
0.100	6.64	0.14

DISCUSSION.

The results of the above experiments on the whole confirm those of earlier workers in this field, who found that the addition of salts depresses the p_H of solutions of amphoteric electrolytes, should this be above the isoelectric point. This phenomenon might be interpreted as being due to change in the activity factor, f_a , of the salts already present, according to the equation:

$$C_H = K [\text{acid}]/f_a [\text{salt of acid}].$$

This explanation does not, however, embrace the phenomena observed in their entirety. Thus, if we accept the existence of zwitterions, the dissociation not only of the carboxyl groups, but also that of part of the amino-groups has to be taken into consideration, when the action of added salts is the resultant of their action on both the acidic and the basic groups of the ampholyte. Next, apart from the activity factor, f_a , other factors, arising from the degree of dissociation of the salts added, may come into play, particularly in the case of salts giving rise to multivalent cations. Thirdly, at certain p_H values the salt may, as a result of hydrolytic dissociation, give rise to hydroxides possessing a smaller f_a than that of the base added to render the solution alkaline. Finally, the solubility of the salts formed with the ampholyte should

be taken into consideration; thus at certain p_H values the addition of calcium or magnesium chloride to ovalbumin solutions gives rise to turbidity.

We shall in a future communication examine more closely the factors capable of reducing the p_H of ampholyte solutions on addition of electrolytes; in this paper we desire to draw attention to the following facts.

The results obtained by adding different concentrations of electrolytes (0.0025–0.025 M) are very different from those observed in solutions of crystalloids. Michaelis and Krüger's [1921] experiments on the effect of salts on the p_H of solutions of phosphoric acid, glycine, *etc.*, have shown clearly that, within the limits of concentration of salt studied, these changes are quite inconsiderable and are proportional to the concentration of salt added. Our experiments show on the other hand that salts giving rise to bivalent cations or anions provoke, within certain p_H limits, considerable change in p_H , which is not proportional to the concentration of salt added. Thus, using 1 % gelatin solution at an initial p_H 5.9, the addition of calcium chloride to a concentration of 0.0025 M lowers the p_H by 0.21, the addition of a second portion of salt to 0.005 M lowers the p_H by only 0.04, whilst on addition of a third portion the difference is barely 0.025. Substantially the same results are obtained using magnesium chloride (first portion 0.12, second 0.08, third 0.03) or sulphate or sodium sulphate. This phenomenon is least marked with potassium chloride, in which case a concentration of 0.1 M , *i.e.* 40 times as great as for calcium chloride, has to be employed in order to achieve the same effect.

It will readily be seen from the above that the results obtained are quite different from those found for glycine, addition to which of calcium chloride to 0.0025 M reduces the p_H by only 0.02.

The reason for the different behaviour of univalent and bivalent ions may lie in the greater effect of the latter on the activity factor of the ampholyte, as follows from Eucken's [1930] formula:

$$\log f_a = -0.354 n_i^2 \sqrt{\Gamma},$$

where $\Gamma = \sum c_i n_i^2$, c_i is the ionic concentration, and n_i is the charge on the ions; the term n_i occurs twice.

A second reason for this difference may be differential aggregation of ions on the surface of the colloid, as a result of adsorption. The researches of Freundlich and of his collaborators led him to the conclusion that, even assuming equal adsorption of uni-, bi- and ter-valent ions, the effect will be the greater the higher the valency of the ions. It appears to us that the fact that the valency factor occurs twice in the formula expressing the activity factor can only partially explain the difference in the effect of varying the concentration of potassium chloride on the one hand and of calcium and magnesium chlorides and of sodium and magnesium sulphates on the other.

It is our opinion that the difference in the action of salts on the p_H of colloidal solutions of gelatin, ovalbumin, and peptone, in comparison with

their action in the case of crystalloids, is a consequence of the sorption of ions on the surfaces of the colloidal particles. This view is supported by the shape of the curves obtained, which resemble adsorption isotherms. The ions undergoing sorption are concentrated in the hydrosphere of the colloidal particles; since it is in this region that compounds responsible for the p_H of the solution, such as albuminates, are present, it would follow that the action of added salts should be greater in heterogeneous than in homogeneous systems. Should it be desired in such cases to determine $\log f_a$ and Γ , it would be necessary to substitute for C , the total ionic concentration per unit volume, not the concentration calculated for unit volume of the system as a whole, but for unit volume of the hydrosphere of the colloidal particles.

The differential actions of various electrolytes would be a consequence of the different valencies of their ions, and of differences in the extent to which they undergo sorption. Thus univalent ions are possibly only very feebly sorbed, and for this reason the curves obtained are of a similar type to those for solutions of crystalloids, whilst bivalent ions would be more powerfully sorbed. This conception is supported by the results obtained by Białaszewicz [1928] and by his collaborator Zawadzki [1929], who have shown that the percentage of the ions of various electrolytes present in the dispersed phase of the ova of various animals varies greatly according to the valencies of the ions in question. In view however of the circumstance that univalent ions were in every case present in excess in the systems studied by these authors, the above hypothesis requires further experimental confirmation.

If we compare the actions of different salts giving rise to bivalent cations, it will be seen that the action of calcium chloride is very similar to that of magnesium chloride, as is evidenced both by the general similarity of the curves obtained and by the absolute values for reduction of p_H brought about by the presence of equimolar concentrations of both salts. The curves obtained from experiments in which bivalent cations are added to solutions of gelatin, ovalbumin, and peptone are not uniform for each colloid. Thus in the case of albumin a rapid decline in p_H , amounting to 0.21, is observed in concentrations of calcium chloride up to 0.01 M , whilst further addition of this salt up to 0.025 M lowers the p_H by barely 0.03. Gelatin solutions at first exhibit a rise in p_H of 0.30 in 0.005–0.01 M calcium chloride followed by a diminution in p_H amounting in 0.01–0.025 M solutions to about 0.13. The least characteristic curves, and the least resembling adsorption isotherms, are those obtained using peptone solutions, in which case the rapid decline in p_H persists up to 0.02–0.025 M calcium chloride. These differences on the whole support our view as to the fundamental difference in the action of ions on solutions of colloids and of crystalloids, although in all probability they are due partly to differences in the number of buffer groups and in those combining with ions on the surface of the colloidal particles present.

Those experiments performed on systems the p_H of which had been altered by the addition of alkali show that in the vicinity of the isoelectric point the

action of ions is least; as the p_H of the system increases the effect obtained is greater, until at a certain value a maximum effect is observed, after which it again declines. This phenomenon was observed with both gelatin and peptone solutions.

It is of interest that in systems the p_H of which is above the isoelectric point, and in which the colloidal particles should be exceptionally sensitive to differences in the valency of the ions added, salts giving rise to a univalent cation and a bivalent anion, *e.g.* sodium sulphate, have a considerably greater action than have univalent salts, such as sodium or potassium chloride.

The two following biologically important conclusions may be drawn from the above-described experiments. The first follows from the considerable reduction of p_H brought about by the presence in small concentrations of calcium and magnesium ions. The greatest effects are observed for concentrations within the limits 0.005–0.01 *M*. Since a 0.005 *M* solution of calcium chloride is equivalent to 55 mg./100 cc. CaCl_2 , or 19.5 mg./100 cc. Ca, and the calcium content of blood is normally about 10–12 mg./100 cc., it is apparent that small variations from the normal value may exert a certain influence on the p_H of the fluids of the organism. These variations in p_H would depend not on the total calcium content, but on the concentration of this ion in the hydrosphere of the colloidal particles of protein.

The action of calcium ions is, as appeared in the course of further experiments, to a large extent dependent on the presence of sodium and potassium ions, which greatly diminish the magnitude of the effect obtained. Thus 0.01 *M* calcium chloride reduces the p_H of a 1 % gelatin solution at p_H 5.9 by 0.31, whilst in the presence of 0.150 *M* potassium chloride the change is only 0.09.

Our second conclusion concerns enzymic reactions, the velocity of which is a function of the p_H of the medium. It follows from Myrbäck's [1926] work on ptyalin that the optimum reaction for this enzyme is at p_H 6.96, whilst at p_H 5.4 and 8.3 the velocity of reaction is only 50 % of that at p_H 6.96. If now the p_H is reduced from 8.3 to 7.8, a difference of 0.5, the observed hydrolysis will rise from 45 to 70 % of that at optimum p_H , *i.e.* the velocity of reaction will be increased by 56 %. The same reduction of p_H below 6.96 will lead to a corresponding inhibition of reaction. The possibility therefore arises that the velocity of enzymic reactions may to a certain extent be regulated by the presence of small concentrations of multivalent ions in systems containing protein.

Whether, under biological conditions, in the presence of high concentrations of univalent ions, this phenomenon plays any important part in the regulation of the velocity of enzymic reactions is questionable. Experiments are however in progress, with a view to the verification of this postulate, and to the determination of its practical importance in vital processes.

SUMMARY.

1. The addition of various mineral salts changes the p_H of solutions of gelatin, ovalbumin and peptone.
2. The depression of p_H is at a maximum on the alkaline side of the isoelectric point.
3. The action of salts is a function not only of their concentration but also of the valency of their ions.
4. The effect produced by unit concentration of salts is greater at very low than at higher concentrations.

REFERENCES.

- Białaszewicz (1928). *Ann. Physiol.* **4**, 190.
Eucken (1930). *Lehrb. d. chem. Physik* (Leipzig).
Freundlich (1930). *Kapillarchemie* (Leipzig).
Kruyt (1928, 1929). *Kolloid Beih.* **28**, 3, 407; **29**, 396, 413.
Michaelis and Krüger (1921). *Biochem. Z.* **119**, 307.
Mond (1923). *Pflüger's Arch.* **200**, 422.
Myrbäck (1926). *Z. physiol. Chem.* **159**, 1.
Netter (1925). *Pflüger's Arch.* **208**, 16.
Simms (1929). *J. Gen. Physiol.* **12**, 511.
Spiro (1922). *Kolloid Z.* **31**, 345.
Zawadzki (1929). *Acta Biol. Exp. Warsaw*, **4**, 119.

LVIII. THE SELF-SELECTION OF FOOD CONSTITUENTS BY THE RAT.

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OSBORNE and MENDEL [1918] and Mitchell and Mendel [1921] have described experiments in which rats and mice were offered the choice between adequate and inadequate food mixtures. The latter authors summarise their results as follows. "The outcome of this investigation shows in general that in their choice of foods, even between 'synthetic' mixtures which appear to the senses to be essentially alike, rats and mice make selections which are as a rule advantageous for their nutritive condition."

The experiments described below were planned with the aim of ascertaining whether rats would be able to face successfully a similar but probably more complex problem, namely that of choosing for themselves a suitably balanced diet when given access to isolated food components.

In order to simplify the problem as far as possible fats were not made an item of choice for the rats¹. Similarly a fixed supply of vitamins was given, so the rats were only called upon to choose between three variables—proteins, carbohydrates and minerals.

EXPERIMENTAL.

A litter of six albino rats of Wistar origin, consisting of 3 males and 3 females, was used for the experiment. The animals were weaned at 3 weeks, and kept for 1 week on our stock ration (a slightly modified Steenbock [1923] diet) before being transferred to the experimental diet. Two animals of each sex were placed upon the "free choice" regimen, consisting of caseinogen, sucrose and salt mixture (McCollum's 185) offered in separate cups. The remaining male and female served as controls and were given the same food constituents in the form of a mixture, composed of 75 parts sucrose, 25 parts caseinogen, and 4 parts salt mixture. This diet is known to give good growth when supplemented with cod-liver oil and a suitable concentrate of the B vitamins [Evans and Lepkovsky, 1929].

The caseinogen used in the experiment was prepared in the laboratory from crude commercial "casein" by repeated washing with acidulated water in wooden tubs. It contained 86.5 % protein ($N \times 6.25$) on the dry basis.

Each of the six rats received daily 2 drops of cod-liver oil fed by hand,

¹ Synthetic diets almost free from fat are very well utilised by rats and the amount of fatty substance present in ordinary washed caseinogen and in cod-liver oil is more than sufficient to rule out the possibility of the fat-deficiency disease recently described by Burr and Burr [1929, 1930].

and 0.25 cc. of a watery extract of brewer's yeast [Osborne and Wakeman, 1919] equivalent to 0.5 g. of dry yeast (nitrogen content—8 mg. per dose).

The rats were kept in individual round cages on screens and the nutrients were offered in small glass cups fitted with metal hoods to prevent scattering. This however, especially in the case of the salt mixture and dry caseinogen, could not be entirely avoided, but Petri dishes placed under the cages beneath each food cup permitted a satisfactory separation and recovery of the scattered food constituents and a quantitative estimation of the amounts ingested. The food consumption was recorded daily, except on Sundays. On Saturdays a double portion of the nutrients was weighed out in the food cups, and the food intake for 2 days was measured every Monday. Double rations of the vitamins were also given on Saturdays. The food cups containing the various constituents were always placed in the cages in the same order. They were reversed once during the experiment. Towards the close of the experiment the sugar was replaced by rice starch.

The experiment lasted 10 weeks (69 days). In the course of this time the experimental rats Nos. 116 and 117 died after 54 and 61 days respectively, the first with symptoms of peritonitis, while the cause of death of the second was not ascertained.

Weekly averages of the food consumption of the four experimental rats and a chart comparing the average food intake of the experimental and control rats as well as growth curves of the experimental animals and an average growth curve of the two controls are given in Figs. 1, 2 and 3. All the figures are calculated on a water-free basis, the values for protein refer to 100 % protein ($N \times 6.25$). The non-protein solids in caseinogen were included in the "sugar."

At first glance the figures disclose two salient features: firstly the protein intake of the experimental rats is very low, being in every case less than 10 % (with the exception of the premortal periods of rats Nos. 116 and 117 and of one period for rat No. 115), and secondly their growth increase is small. In fact, if the ability to grow is taken as a measure of the success of the experiment, then it must be said that, on the whole, the rats were unable to select a mixture which would surpass or equal in efficiency the prepared mixture based on conventional standards. On a quantitative or caloric basis the proportions selected by the rats are also far from being economic.

Table I gives the total intake of protein, sugar (and later rice starch), salts and Calories (calculated) for the experimental rats and for the controls.

From these figures the number of Calories consumed per g. increase of weight has been calculated for each rat.

Rat	Calories per g. increase
114	49.6
115	31.1
116	65.4
117	43.2
Average for two controls	22.2

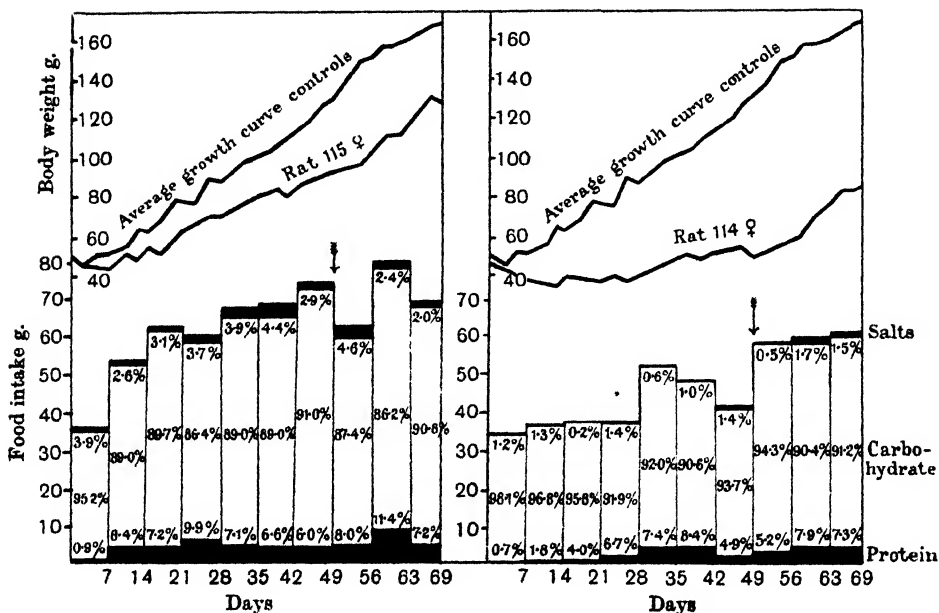


Fig. 1. Weekly food intake of rats 114 and 115.

At ↓ sugar changed to rice starch.

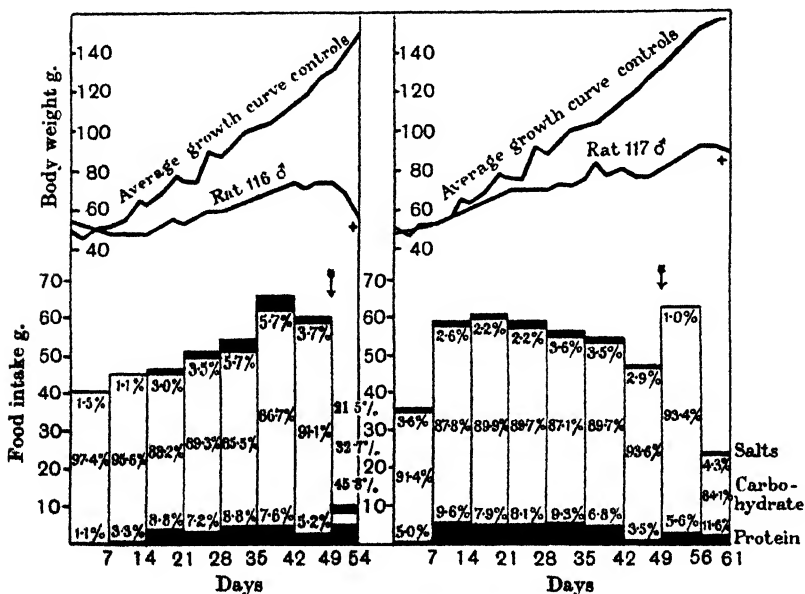


Fig. 2. Weekly food intake of rats 116 and 117.

At ↓ the sugar was changed to rice starch.

When the first seven weekly periods during which all the experimental rats remained alive are taken as a basis, the following average figures are obtained:

	Consumption Calories	Initial weight g.	Weight increase g.	Consumption Calories per g. increase
Control rats	1568	48	82	19.1
Experimental rats	1438	49	26	55.3

A calculation for the last six periods has also been made for rats 114 and 115. It may well be assumed that at this time the rats had become well accustomed to the experimental conditions. Here the figures are more in favour of the experimental rats:

	Consumption: Calories	Initial weight g.	Weight increase g.	Consumption Calories per g. increase
Rat 114	1289	40	43	30
Rat 115	1679	70	59	28.5
Control rats	1891	89	78	24.2

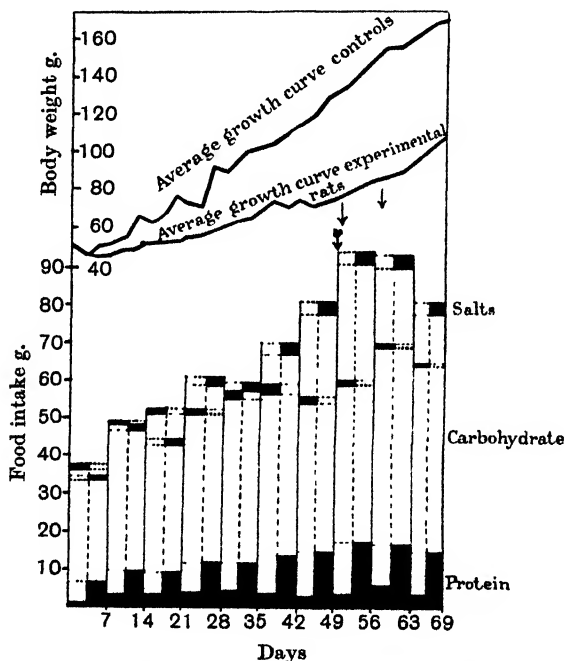


Fig. 3. Comparison of weekly food intake of experimental and control rats.

The left half of each rectangle represents the average food intake of the experimental rats, the right one that of the controls.

The ↓ arrows represent the death of an experimental rat.

The ↴ arrow represents a change from sugar to rice starch.

It must be remembered however that the control animals were heavier than either of the experimental ones and that, according to Hitchcock [1927], "The caloric cost of a gram increase in weight is inversely proportional to the square root of the percentage increase in weight." This of course would

imply that the heavier controls were handicapped when compared with the lighter experimental animals.

It is clearly visible from the curves in Fig. 4 that the animals allowed the choice of food actually consumed more Calories per unit of weight than their

Table I.

Consumption of	Total g.	Daily g.	%	Total Calories	Calories daily
Rat 114. No. of days on experiment: 69; initial weight: 46 g. weight increase: 38 g.					
Protein	26.9	0.39	5.8	110	1.6
Sugar*	432.4	6.27	93.1	1773	25.7
Salts	5.1	0.07	1.1	—	—
Total	464.4	6.73	100.0	1883	27.3
Rat 115. No. of days on experiment: 69; initial weight: 48 g. weight increase: 81 g.					
Protein	48.4	0.70	7.6	198	2.9
Sugar*	565.7	8.20	89.1	2319	33.6
Salts	20.9	0.30	3.3	—	—
Total	635.0	9.20	100.0	2517	36.5
Rat 116†. No. of days on experiment: 49; initial weight: 54 g. weight increase: 22 g.					
Protein	22.7	0.46	6.2	93	1.9
Sugar*	328.1	6.70	90.1	1345	27.4
Salts	13.3	0.27	3.7	—	—
Total	364.1	7.43	100.0	1438	29.3
Rat 117†. No. of days on experiment: 56; initial weight: 47 g. weight increase: 40 g.					
Protein	31.0	0.55	7.2	127	2.3
Sugar*	390.5	6.97	90.2	1601	28.6
Salts	11.3	0.20	2.6	—	—
Total	432.8	7.72	100.0	1728	30.9
AVERAGE FOR CONTROL RATS.					
No. of days on experiment: 69; initial weight: 48 g. weight increase: 118 g.					
Protein	131.7	1.91	19.8	540	7.8
Sugar*	508.3	7.37	76.6	2084	30.2
Salts	24.1	0.35	3.6	—	—
Total	664.1	9.63	100.0	2624	38.0

* And, later, rice starch.

† The last incomplete premortal period is omitted.

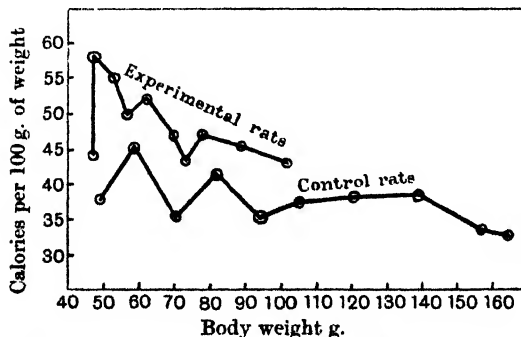


Fig. 4. Consumption of Calories per 100 g. of weight. Weekly averages.

The curve for the experimental rats represents an average for four rats for the first seven weekly periods, for three for the eighth period, and for two for the ninth and tenth periods.

control mates when equal weights are compared, while their growth was at the same time far inferior.

The results are much more favourable for the experimental rats when their weight increments are evaluated on the basis of protein intake. The weight increase per g. of protein ingested can easily be calculated from the figures given in Table I with the following result.

Rat	Weight increase per g. protein
114	1.41
115	1.67
116	0.97
117	1.27

The average protein intake for the four experimental rats for the first 7 weekly periods is 23.5 g. (6.5 % of the total food intake), while the weight increase for the same period amounts to 26 g., a ratio of 1.08. For the last six periods (42 days) the values are:

Rat No.	Protein consumed <i>P</i> (g.)	% of total food intake	Weight increase <i>W</i> (g.)	Ratio <i>W/P</i>
114	21.9	6.7	43	1.96
115	33.1	7.6	59	1.78

It is obviously not possible to compare the experimental rats with the controls in this respect, as the food of the latter contained a rather large percentage of protein, probably well over the requirements for growth at "normal" rate.

On the other hand the values obtained above are nothing else but the biological value of the food protein, caseinogen in this case, calculated according to the method of Osborne, Mendel and Ferry [1919] and may be compared with data found in the literature for caseinogen.

These authors obtained a value for caseinogen of 1.45 at a 9.3 % level in an experiment of 4 weeks' duration and in another experiment a ratio of 1.76 (11 weeks) and 1.77 (4 weeks) at a 9.2 % level. Hoagland and Snider [1926] found a value of 1.98 for caseinogen at a 10.0 % level in a 30 days' experiment. These values refer to levels higher than those encountered in this experiment and, as the maximal values for caseinogen are found, according to Osborne, Mendel and Ferry [1919], at the 12 % level and decrease in both directions, the present figures are at least as good as those reported in previous observations. This finding certainly confirms the obvious supposition that the low level of protein intake was the first limiting factor in the diet freely selected by the experimental rats, but does not supply any additional information as to the reason for this rather surprising choice. It would seem however reasonable to assume, judging from these results, that the poorer growth of the experimental animals was not due to other causes connected with the free selection of their food, such as daily variation of the ratio of various constituents and so on.

As contrasted with the low intake of protein, the voluntary consumption by the "free-choice" rats of the mineral components of the diet in the form

of salt mixture did not differ greatly from the accepted standards, with the exception of rat No. 114. This animal consumed on the average only 1.1 % salts or one-third as much as the remaining three experimental rats. It does not seem however that this low intake of minerals limited the growth of rat No. 114. The protein was here probably the first limiting factor.

Owing to the low voluntary ingestion of protein, sugar formed over 90 % of the diet of the experimental rats (90.6 % as an average for the first 7 weekly periods). It is well known that rats eat sugar very readily and it was thought possible that the factor of taste might have induced the rats to satisfy their caloric requirements almost entirely with a highly palatable carbohydrate to the detriment of the more or less insipid protein. The sugar was therefore replaced by rice starch at the beginning of the 8th weekly period. This change did not however exert the slightest influence on the intake of protein, the only noticeable result being a short temporary drop in the consumption of the carbohydrate.

As already stated the order in which the food cups were placed in the cages of the experimental rats was changed once in the course of the experiment. This had no noticeable effect on the intake of the various food constituents, even on the day of the change.

DISCUSSION.

Perhaps the most noteworthy fact in the above experiment is the voluntary limitation by the experimental rats of their protein intake well below the standards accepted as normal or necessary for the rat. At first sight this result might certainly be interpreted as giving "natural" evidence in favour of low-protein diets and as an objective argument confirming the views of certain students of nutrition. Any such reasoning however would lose its point in face of the poor growth and performance of rats voluntarily subsisting on such diets. The inferiority of the experimental animals as compared with controls consuming a diet richer in protein makes an explanation of their choice highly interesting and desirable but at the same time very difficult. A simple explanation could be offered on the assumption that caseinogen *per se* is distasteful to the rats and that the dislike for this nutrient causes them to limit the intake. It must be remembered however that, when given no choice, the control rats ingested without difficulty adequate amounts of a diet containing 3 times as much caseinogen, and that Osborne and Mendel [1921, 1, 2], Osborne *et al.* [1927], Reader and Drummond [1924-5] and others found quite satisfactory food consumption on diets consisting almost entirely of caseinogen. It is also known that, in search for necessary nutrients, animals subsisting on deficient or faulty diets will consume substances which they would not touch under normal conditions, and which in all probability are distasteful; for example the coprophagy of rats fed on "synthetic" diets, an almost constant phenomenon in all laboratories, the pica or bone-eating disease of cattle and many other examples of depraved appetite. Though a particular

dislike for caseinogen cannot easily be ruled out, it does not seem to afford a sufficient explanation for the rather noteworthy failure of the experimental rats.

Mitchell and Mendel [1921], when offering rats the choice between two diets, one consisting of ground whole corn, with a protein ($N \times 6.25$) content of 9.5 %, the other of meat meal, prepared "by thoroughly drying lean round-steak and then grinding to a meal" and containing 79 % protein ($N \times 6.25$), while a salt mixture was freely accessible in both cases, observed that "contrary to what might have been expected the younger animals selected a smaller proportion of protein in their diets than did the older ones. On the other hand none of the rats chose an excessively 'high-protein' diet although an abundance of the meat meal was always available."

One of the younger animals (rat 21) referred to by Mitchell and Mendel consumed a diet containing only 12 % of Calories in the form of protein; in other words it hardly touched the meat meal. The other (rat 22) consumed 19 % of protein Calories. Both these values are well above the levels ingested by the rats in the present experiment, and, moreover, the rats of Mitchell and Mendel achieved much better growth, rat 21 gaining approximately 85 g. and rat 22 approximately 110 g. in 8 weeks. It must be remembered however that the lowest possible protein intake was for these rats higher than the level voluntarily selected by the animals in the present experiment. Whether the results obtained by Mitchell and Mendel give a truthful picture of the protein requirements of young growing rats or whether they were to any degree complicated or caused by an unequal distribution of vitamins, especially vitamin B, in the two natural foods employed by these authors remains to be answered. Taken as they are they nevertheless point in the same direction as the rather extreme results set forth in this paper.

When the present experiments were planned it was thought that their outcome might throw some light on the much debated question of the optimal level of protein intake and also on its variation in relation to growth (change in nutritive ratio). It seems however that any definite conclusions regarding the "natural" or "instinctive" (whatever these words may imply) protein requirements of young growing rats must be withheld until further experiments with different suitable proteins are instituted.

It has been taken for granted that free choice of regimen is a reliable guide for animals living under normal conditions. It certainly is well worth while to ascertain whether nature is not at a loss in conditions so highly artificial as described in the present paper.

SUMMARY.

1. Young growing rats, offered sucrose (later changed to rice starch), caseinogen and a salt mixture in separate dishes and allowed to make their own choice of these food constituents, consumed on an average only 6.5 % of protein.

2. Changing the sucrose to rice starch did not affect the intake of protein.
3. Two of the experimental rats died in the course of the experiment.
4. Litter mate controls which were given a ready-mixed diet consisting of the same ingredients and containing about 20 % of protein made much greater gains in weight.
5. All rats received equal vitamin supplies, fed separately by hand.

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REFERENCES.

- Burr and Burr (1929). *J. Biol. Chem.* **82**, 345.
—— ——— (1930). *J. Biol. Chem.* **86**, 587.
Evans and Lepkovsky (1929). *J. Biol. Chem.* **83**, 269.
Hitchcock (1927). *Amer. J. Physiol.* **83**, 28.
Hoagland and Suider (1926). *J. Agric. Res.* **32**, 679.
Mitchell and Mendel (1921). *J. Biol. Chem.* **58**, 211.
Osborne and Mendel (1918). *J. Biol. Chem.* **35**, 19.
—— ——— (1921, 1). *Proc. Soc. Exp. Biol. Med.* **18**, 167.
—— ——— (1921, 2). *Proc. Nat. Acad. Sci.* **7**, 157.
—— Mendel and Ferry (1919). *J. Biol. Chem.* **37**, 223.
—— ——— Park and Winternitz (1927). *J. Biol. Chem.* **71**, 317.
—— and Wakeman (1919). *J. Biol. Chem.* **40**, 383.
Reader and Drummond (1924-5). *J. Physiol.* **59**, 472.
Steenbock (1923). *Science*, **58**, 449.

LIX. A STUDY OF THE NITROGEN BALANCE IN VITAMIN B₂ DEFICIENCY IN THE RAT.

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It was reported in a previous communication [Kon, 1929] that rats kept on a diet deficient in vitamin B₂ show an increased carbon/nitrogen ratio in the urine as compared with individual controls receiving equal amounts of a diet considered to be complete. No such increase was noted in rats consuming a ration deficient in vitamin B₁.

The present paper deals with a quantitative study by the individual control method of the nitrogen metabolism of rats subsisting on diets low in the heat-stable factor (or factors) of yeast.

EXPERIMENTAL.

A litter of six rats of Wistar stock, bred in the laboratory on a slightly modified stock diet of Steenbock [1923] was used for the experiment. They were weaned at 21 days and, after a week on the stock diet, were run out for 17 days on our basal vitamin B-deficient diet 60 (identical with diet 540 of Evans [Kon, 1929]) and consisting of caseinogen (extracted with acidulated water) 25 parts, commercial cane-sugar 75 parts, and Steenbock's salt mixture No. 40, 4 parts. At the end of this time the litter was divided into three pairs, one member of each pair acting as an individual control to its mate. This method has been more amply described in earlier papers by Kon and Drummond [1927] and Kon [1929]. The animals were placed separately in modified Mitchell's cages [Kon, 1928] and offered the following diet: caseinogen (extracted with acidulated water) 10 parts, rice starch 70 parts, butter-fat 15 parts, and salts (Steenbock's mixture No. 40) 5 parts. The diet contained 1.5 % N and 10 % moisture. A quantity of it sufficient for the whole experiment was prepared and kept in cold storage at - 2°. This diet was selected for several reasons. Firstly in order to avoid spilling as completely as possible and to measure the food intake with a sufficient accuracy it was necessary to offer the food mixed with water to a paste. For this reason rice starch had to be used instead of sugar. Secondly it was thought best for the purpose of the experiment to select a diet low in nitrogen, and containing it at a concentration similar to those used in experiments on the biological value of proteins. Should any deviations from the normal path of nitrogen metabolism manifest themselves in the course of vitamin B₂ deficiency, they would be

most easily detected at such a level of nitrogen intake that most of it, under normal conditions, is used for repair of body tissues and for growth and a small percentage only, if any, burnt as such to cover the caloric requirements. The butter improved the texture of the diet and at the same time supplied vitamins A and D.

The experimental rats were given daily 0.4 cc. of an alcoholic extract from rice bran¹ equivalent to 3.2 g. of bran. This extract was shown in a special experiment to be lacking in vitamin B₂ (and possibly in some other factors supplied in autoclaved yeast). The control animals received an addition of 0.3 g. of dried brewer's yeast of tested potency. In order to equalise the intake of nitrogen an amount of purified caseinogen equivalent in nitrogen content to 0.3 g. of the yeast was placed daily in the porcelain dishes containing the rice bran extract for the experimental animals. The food was mixed with a little water to a pasty consistency to avoid scattering.

For the first four days the food was given to all rats *ad lib*. On the fifth day the residues left in the food cups by the experimental rats were dried at 105° to constant weight, the food intake of each experimental rat was measured and an equivalent amount of the diet was weighed out for the corresponding individual control. The dry residue was discarded from the cups of the experimental animals and fresh food was given slightly in excess of the intake. This procedure was from now on repeated daily, so that the controls were always receiving an amount of food equivalent to that eaten by their mates in the course of the preceding 24 hours. There was practically no scattering and the controls always consumed all the food offered. The yeast, rice bran extract and caseinogen were very readily taken.

The collection of urine and faeces was started after one week of quantitative feeding. This, as well as the analysis of the excreta, was carried out exactly as described in an earlier paper [Kon, 1928]. The rats were weighed once a week, the control animals 24 hours after the experimental ones. The actual metabolic experiment involved five separate collection periods and lasted 36 days, while the quantitative feeding was continued for a week longer (and started a week earlier). The first period lasted 7 days, the following ones 6 days each. At the conclusion of every period the rats were weighed and examined. To avoid any danger of nitrogen losses in the course of handling, an interval of 24 hours separated the periods.

Food consumption.

The food was weighed with an accuracy of 0.01 g. The residues left by the experimental rats were dried for 4–5 hours in an electric oven at 105°

¹ Prepared by extracting rice bran (supplied by Messrs Polski Przemysł Ryżowy in Gdynia) for three days with 25 % by weight alcohol, in the proportion of 4 litres to the kg., concentrating the extract *in vacuo* to a thick syrup, adding a sufficient amount of 95 % alcohol to make the concentration 80 % by weight, decanting the fluid, dissolving the residue in a small volume of hot water, bringing the concentration again to 80 % alcohol, combining the extracts and evaporating them *in vacuo* until 1 cc. is equivalent to 8 g. of the original bran.

until a constant weight was attained. The moisture content of the diet was checked at frequent intervals and was found to remain constant. As the food consumption of the control rats was quite quantitative and the scattering by all the animals negligible (while any food scattered could be easily recovered from the filter-paper on the bottom of a cage), it is felt that the daily difference between the food (diet) intake of a control rat and of its experimental mate did not exceed 0.03 g., the variations being obviously in both directions.

In addition to the diet every control rat consumed 0.3 g. of dried brewer's yeast containing 9.1 % water and 8.81 % N, while the experimental animals received 0.19 g. of caseinogen (with the exception of the first 4 days of the first period, when they were given 0.2 g.) of the same water content and 13.8 % N. The experimental animals were given in addition 0.4 cc. daily of the rice bran extract, containing 2 mg. of nitrogen and 0.164 g. of solid matter, mostly of a carbohydrate nature. This means that the experimental rats consumed daily slightly over 0.06 g. (0.064 g.) of solid matter more than did their controls.

General appearance of rats.

The experimental rats displayed progressively symptoms usually associated with a lack of vitamin B₂, namely rough coats, coagulated blood on paws and vibrissae and urine-stained abdominal fur. The eyes were not involved. One rat (No. 154) had a mild stomatitis. In contrast all the controls were quite normal in appearance and had normal sleek fur.

Weight curves of rats.

In every case the control rats made better gains than their experimental mates. Table I gives the weights at the beginning and end of the quantitative feeding period, while average growth curves for both sets are presented in Fig. 1.

During the whole of the experimental period every experimental rat con-

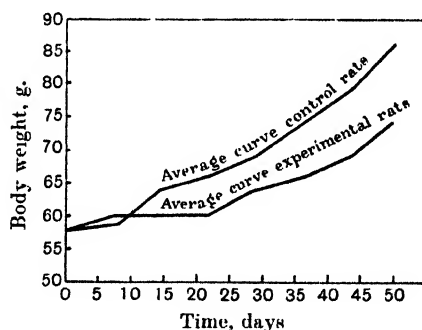


Fig. 1. Average growth curves of experimental and control rats.

Table I.

Rat no.	Sex	Initial weight g.	Final weight g.	Increase g.	Difference	Remarks
153	♀	56	74	18	+ 21	Experimental Control to 153
152	♀	49	88	39		
154	♂	65	75	10	+ 11	Experimental Control to 154
155	♂	65	86	21		
156	♂	56	72	16	+ 7	Experimental Control to 156
151	♀	63	86	23		

Table II. Nitrogen balance.

Period	Rat no.	Average weight		Food intake			Caseinogen intake			Yeast intake			Extract intake			Total intake			N in urine mg.	N in faeces mg.	Total N excreted mg.	N balance mg.
		g.	mg.	g.	mg.	N	g.	mg.	N	g.	mg.	N	g.	mg.	N	g.	mg.	N				
I	153 E	59	31.45	524	1.24	189	—	—	—	1.91	185	—	1.15	14	33.84	727	32	663	+ 64	+ 230		
	152 C	59	31.45	524	—	—	—	—	—	—	—	—	—	—	33.36	709	65	479	+ 16	+ 9		
	154 E	63	26.37	439	1.24	189	—	—	—	1.91	185	—	1.15	14	28.76	642	37	626	+ 58	+ 119		
	155 C	62	26.37	439	—	—	—	—	—	—	—	—	—	—	28.28	624	530	615	+ 9	+ 9		
	156 E	58	31.18	520	1.24	189	—	—	—	1.91	185	—	1.15	14	33.57	723	40	665	+ 58	+ 119		
II	151 C	62	31.18	520	—	—	—	—	—	—	—	—	—	—	33.09	705	65	586	+ 58	+ 119		
	153 E	59	26.72	445	1.03	157	—	—	—	1.64	159	—	0.98	12	28.73	614	50	615	+ 79	+ 79		
	152 C	63	26.72	445	—	—	—	—	—	—	—	—	—	—	28.36	604	467	58	525	+ 50	+ 50	
	154 E	63	23.61	393	1.03	157	—	—	—	1.64	159	—	0.98	12	25.62	562	481	31	512	+ 46	+ 46	
	155 C	65	23.61	393	—	—	—	—	—	—	—	—	—	—	25.25	552	473	33	506	+ 46	+ 46	
III	156 E	59	24.42	407	1.03	157	—	—	—	1.64	159	—	0.98	12	26.43	576	547	48	595	+ 19	+ 19	
	151 C	65	24.42	407	—	—	—	—	—	—	—	—	—	—	26.06	566	465	38	503	+ 63	+ 63	
	153 E	62	26.78	446	1.03	157	—	—	—	1.64	159	—	0.98	12	28.79	615	442	54	496	+ 119	+ 119	
	152 C	67	26.78	446	—	—	—	—	—	—	—	—	—	—	28.42	605	415	74	489	+ 116	+ 116	
	154 E	63	21.24	354	1.03	157	—	—	—	1.64	159	—	0.98	12	23.25	523	457	28	485	+ 38	+ 38	
IV	155 C	67	21.24	354	—	—	—	—	—	—	—	—	—	—	22.88	513	400	52	452	+ 61	+ 61	
	156 E	62	23.69	395	1.03	157	—	—	—	1.64	159	—	0.98	12	25.70	564	429	45	474	+ 90	+ 90	
	151 C	68	23.69	395	—	—	—	—	—	—	—	—	—	—	25.33	554	421	75	496	+ 58	+ 58	
	153 E	67	24.45	407	1.03	157	—	—	—	1.64	159	—	0.98	12	26.46	576	468	38	506	+ 70	+ 70	
	152 C	73	24.45	407	—	—	—	—	—	—	—	—	—	—	26.09	560	392	49	441	+ 125	+ 125	
V	154 E	65	22.78	380	1.03	157	—	—	—	1.64	159	—	0.98	12	24.79	549	418	35	453	+ 96	+ 96	
	155 C	70	22.78	380	—	—	—	—	—	—	—	—	—	—	24.42	539	415	60	475	+ 64	+ 64	
	156 E	63	23.48	391	1.03	157	—	—	—	1.64	159	—	0.98	12	25.49	560	442	38	480	+ 80	+ 80	
	151 C	71	23.48	391	—	—	—	—	—	—	—	—	—	—	25.12	550	422	46	468	+ 82	+ 82	
	153 E	69	23.08	385	1.03	157	—	—	—	—	—	—	0.98	12	25.09	554	431	54	485	+ 69	+ 69	
V	152 C	79	23.08	385	—	—	—	—	—	1.64	159	—	—	—	24.72	544	356	46	402	+ 142	+ 142	
	154 E	68	21.27	354	1.03	157	—	—	—	—	—	—	0.98	12	23.28	523	343	34	377	+ 146	+ 146	
	155 C	76	21.27	354	—	—	—	—	—	1.64	159	—	—	—	22.91	513	343	54	397	+ 116	+ 116	
	156 E	65	20.30	338	1.03	157	—	—	—	—	—	—	0.98	12	22.31	507	425	28	453	+ 54	+ 54	
	151 C	76	20.30	338	—	—	—	—	—	1.64	159	—	—	—	21.94	497	377	62	439	+ 58	+ 58	

* Assumed; the determination was lost.

Table III. Complete nitrogen balance (36 days).

Rat no.	Initial weight g.	Final weight g.	Weight increase g.	Food intake			Caseinogen intake			Yeast intake			Extract intake			Total intake			Excreted N	Balance N
				g.	mg.	N	g.	mg.	N	g.	mg.	N	g.	mg.	N	g.	mg.	N		
153 E	58	71	13	152.59	2543	6.21	947	951	9.82	951	951	9.82	951	951	9.82	164.67	3562	3189	+ 273	+ 273
152 C	56	81	25	152.59	2543	—	—	—	—	—	—	—	—	—	—	162.41	3494	2696	+ 799	+ 799
154 E	64	69	5	135.22	2253	6.21	947	951	9.82	951	951	9.82	951	951	9.82	147.30	3272	2863	+ 409	+ 409
155 C	61	78	17	135.22	2253	—	—	—	—	—	—	—	—	—	—	145.04	3204	2837	+ 347	+ 347
156 E	58	67	9	142.61	2377	6.21	947	951	9.82	951	951	9.82	951	951	9.82	154.69	3396	3089	+ 307	+ 307
151 C	60	78	18	142.61	2377	—	—	—	—	—	—	—	—	—	—	152.43	3328	2899	+ 439	+ 439

sumed 3.36 g. solid matter (mostly assimilable carbohydrates) more than his control mate and, nevertheless, made a smaller gain.

Nitrogen balance.

Table II gives the intake and excretion of nitrogen during the five metabolic periods.

It will be recalled that between each metabolic period there was a gap of 24 hours when no collection of urine or faeces was made, the food intake however being recorded in the usual way. In order to calculate the nitrogen balance over the whole duration of the metabolic experiment, it was therefore necessary to include data for the nitrogen excreted in urine and faeces on the days when no actual measurements were made. These figures have been calculated on the probably correct assumption that the nitrogen excretion on the "gap" day would stand in the same ratio to the nitrogen intake as the actual figures obtained for the immediately preceding period. The results are presented in Table III.

An inspection of Tables II and III shows that for the entire period two control rats, Nos. 152 and 151, retained more nitrogen than their experimental mates, while the reverse was true for the control rat No. 155 (which nevertheless gained more in weight than his experimental mate). When the 30 separate measurements carried out in the course of the metabolic experiment are compared in 15 pairs it becomes evident that the control rats were favoured in 9 cases, while the experimental ones retained more nitrogen in 6. It will be noticed that the differences tend to become less marked as the experiment progresses, the values for the second and third pairs of rats running very closely together.

The excretion of faecal nitrogen is almost always higher in the controls (with 2 exceptions), probably due to the consumption of yeast and the production of more bulky faeces [Mitchell, 1924, 1]. It was thought that a clearer picture of the nitrogen metabolism of both groups of rats might be obtained if the biological value of the ingested nitrogen were calculated from the available data by means of the method of Mitchell [1924, 1; Mitchell and Carman, 1926, 2]. Such a calculation necessitates the use of figures for the "metabolic faecal nitrogen" and for the "endogenous urinary nitrogen," usually obtained by measuring the nitrogen excretion of rats during periods of nitrogen-free or of low egg-nitrogen diet. It was not thought safe to include such periods in the work described here, in order not to vitiate the results. It has therefore been necessary to introduce the following assumed factors, based on values obtained previously by Kon [1928] and by Mitchell [1924, 1; Mitchell and Carman, 1926, 2; Mitchell, Beadles and Kruger, 1927], for rats of approximately the same weight as those used in the present experiment. The assumed values are: 2 mg. of metabolic faecal nitrogen per g. of food consumed and 25 mg. of endogenous urinary nitrogen per day per 100 g. of weight. The results of such calculation are presented in Table IV.

Table IV. "*Biological value*" of the ingested nitrogen calculated according to Mitchell.

Period	Rat no.	Average weight	Food intake		Faecal N mg.	Body N in faeces mg.	Food N in faeces mg.	Ab-sorbed N mg.	N in urine mg.	Body N in urine mg.	Food N in urine mg.	Food N re-tained mg.	Bio-logical value
			g.	N mg.									
I	153 E	59	33.84	727	32	68	0	727	631	103	528	199	27
	152 C	59	33.36	709	65	67	0	709	411	103	311	398	56
	154 E	63	28.76	642	37	57	0	642	589	110	479	163	25
	155 C	62	28.28	624	65*	56	9	615	550	108	442	173	28
	156 E	58	33.57	723	40	67	0	723	625	101	524	199	27
	151 C	62	33.09	705	65	66	0	705	521	108	413	288	41
II	153 E	59	28.73	614	50	57	0	614	565	88	477	137	22
	152 C	63	28.36	604	58	57	1	603	467	94	373	230	38
	154 E	63	27.62	562	31	51	0	562	481	94	387	175	31
	155 C	65	25.25	552	33	50	0	552	473	97	376	176	32
	156 E	59	26.43	576	48	53	0	576	547	88	459	117	20
	151 C	65	26.06	566	38	52	0	566	465	97	368	198	35
III	153 E	62	28.79	615	54	58	0	615	442	93	349	266	43
	152 C	67	28.42	605	74	57	17	588	415	100	315	273	46
	154 E	63	23.25	523	28	46	0	523	457	94	363	160	31
	155 C	67	22.88	513	52	46	6	507	400	100	300	207	41
	156 E	62	25.70	564	45	51	0	564	429	93	336	228	40
	151 C	68	25.33	554	75	51	24	530	421	102	319	211	40
IV	153 E	67	26.46	576	38	53	0	576	468	100	438	208	36
	152 C	73	26.09	566	49	52	0	566	392	109	283	283	50
	154 E	65	24.79	549	35	50	0	549	418	97	321	228	41
	155 C	70	24.42	539	60	49	11	528	415	105	310	218	11
	156 E	63	25.49	560	38	51	0	560	442	94	348	212	38
	151 C	71	25.12	550	46	50	0	550	422	106	316	234	42
V	153 E	69	25.00	551	54	50	4	550	431	103	328	222	40
	152 C	79	24.72	544	46	49	0	544	356	118	238	306	56
	154 E	68	23.28	523	34	46	0	523	343	102	241	282	54
	155 C	76	22.01	513	54	46	8	505	343	114	229	276	54
	156 E	65	22.31	507	28	45	0	507	425	97	328	179	35
	151 C	76	21.94	497	62	44	18	479	377	114	263	216	45

* Assumed.

Table V gives the average biological values of the ingested nitrogen for all periods for the experimental and control rats. Out of the 15 pairs of

Table V.

Rat no.	Biological value	
153 E	34	
152 C	49	
154 E	36	General average for experimental rats: 34
155 C	39	
156 E	32	General average for control rats: 43
151 C	41	

biological values obtained 3 give equal values for both rats while the remaining 12 are in favour of the control rat, though in 5 cases the difference does not exceed 4, well within the experimental error.

It will be remembered that while the experimental rats derived their

nitrogen almost exclusively from caseinogen, the diet of the control rats contained a considerable proportion (over one-fourth of the total crude protein) of yeast proteins ($N \times 6.25$) in addition to caseinogen. It would be obviously quite simple to use a watery extract from yeast [Osborne and Wakeman, 1919] instead of yeast as a source of water-soluble vitamins for the control rats, but in the light of recent work [Kennedy and Palmer, 1928; Hunt, 1928; Sure, 1928] it seems doubtful whether such extracts contain all the factors originally present in yeast. Preliminary experiments carried out by the author (unpublished work) seemed to confirm this opinion and it was therefore thought safer to avoid the use of extracts.

It might be argued that a supplementary relation exists between caseinogen and yeast nitrogen, yielding a mixture of higher biological value than caseinogen itself and that the differences in weight, retention of nitrogen and biological value of the ingested nitrogen between the experimental rats and the controls might be wholly explained on this basis. The author is not aware of any published work on the biological value of such a combination or of yeast at a 12 % level (*i.e.* at approximately the level of protein used in the present work). He (unpublished experiments) has measured by the method of Osborne, Mendel and Ferry [1919] the biological value of the nitrogen of yeast and has found values of 1.48, 1.31 and 1.36 when fed at a level ($N \times 6.25$) of 8.4, 10.0 and 12.1 % respectively. Osborne, Mendel and Ferry found a maximal value for caseinogen of 2.25 when fed at 12 % level. It does not seem likely that this value would be raised by the addition of yeast. Admittedly this reasoning can only by indirect inference be applied to calculations based on Mitchell's method of evaluating the biological value of proteins. A more direct proof is found in the behaviour of rats Nos. 154 and 155. In this pair the control rat retained less nitrogen than the experimental one, while the calculated biological values of the ingested nitrogen are practically identical for both rats. Nevertheless the control animal gained 21 g. in weight and the experimental only 10 g., a difference equal to 17 % of the initial weight of the rats. It will be also recalled that in the previous communication [Kon, 1929] a distinct difference was reported between the weights of vitamin B_2 -deficient and control rats while the former consumed a diet containing a high percentage of caseinogen (25 %), certainly more than necessary for optimal growth if taken in adequate amounts. At such level an increase of the efficiency of caseinogen by the addition of another protein is hardly to be expected.

The biological values calculated by the method of Mitchell for the experimental and control rats are both very markedly below the values found for caseinogen by Mitchell [1924, 2] at a 5 % and by Kon [1928] at an 8 % level, namely 71 and 68 respectively. Mitchell and Hamilton [1929, p. 537] believe that a variable food intake for rats of approximately the same size exerts no effect on the biological values obtained, provided the amount of food consumed is adequate for the maintenance of weight. Mitchell, Beadles and Kruger [1927] noted extremely low figures (zero in several cases) for rats

consuming inadequate amounts of a diet containing pork cracklings as a source of protein, while another group of rats consuming the food better gave a value of 25. The former rats lost weight. In the present experiment all the rats gained weight at a very slow rate, they should consequently, according to Mitchell and Hamilton [1929] give normal biological values (or at least the control rats). There is no doubt that the food intake was subnormal and that the control rats were prevented from growing at a more rapid rate only because their food was limited. It seems possible that at such low levels of food intake a part of the ingested protein may be used for energy purposes while the remainder is used for maintenance and even allows for growth at very slow rate. It is difficult to account otherwise for the very substantial difference between the biological values reported now and found previously for caseinogen.

A calculation of the biological value of the ingested nitrogen according to the method of Osborne, Mendel and Ferry [1919] can be made for the whole period of quantitative feeding covering 52 days. The following values are obtained (Table VI).

Table VI.

Rat no.	No. of days on diet	Consumption of		Gain in weight g.	Biological value
		Food	Protein (N \times 6.25)		
153 E	52	236.68	32.09	18	0.56
152 C	52	233.32	31.41	39	1.24
154 E	52	212.25	29.54	10	0.34
155 C	52	208.88	28.86	21	0.73
156 E	52	224.02	30.77	16	0.52
151 C	52	220.66	30.10	23	0.76

The values obtained for the experimental rats are markedly lower than those found for the control animals. The difference is much more marked than when the biological value of the ingested protein is calculated according to the method of Mitchell. This result is readily understood in view of the very significant differences in weight gains between the two sets of rats. All the values are much below the figures cited previously for caseinogen fed under usual conditions at approximately the same level. This is due to the insufficient food intake.

Positive nitrogen balances have been obtained in the present paper for all the rats studied. If all of the retained nitrogen was used for building protoplasmic tissue then every gram of nitrogen should cause a gain in weight of 31.25 g. on the assumption that the deposited tissue would contain approximately 20 % protein (N \times 6.25).

Table VII gives values calculated on this basis together with the actual gains made by the rats during the metabolic periods.

While for two experimental rats (Nos. 153 and 156) and for one control (No. 152) the calculated values coincide well with the actual gains, the nitrogen retained by rat No. 154 must have been used also for purposes other than the

Table VII.

Rat no.	N retained mg.	Calculated gain in protoplasm	Actual gain
		g.	g.
153 E	373	11.7	13
152 C	799	24.9	25
154 E	409	12.8	5
155 C	347	10.8	17
156 E	307	9.6	9
151 C	439	13.7	18

formation of body protein, and the two control rats, Nos. 155 and 151, would have deposited fat in addition to the formation of the calculated amount of protoplasmic tissue. The only justifiable conclusion is that the gains made by the experimental rats and by the controls were of different composition.

DISCUSSION.

The results here reported were obtained with only a small group of animals (3 pairs) and, though they were rigidly controlled, great caution must be exercised in order not to draw unwarranted conclusions.

Reviewing the evidence on hand the following points seem to deserve special attention.

1. All three control animals made better weight gains than their experimental mates though the former consumed slightly less food. This observation is in agreement with earlier work [Kon, 1929] where, in a similarly controlled experiment, all six controls made better gains than their mates consuming a diet deficient (or low) in vitamin B₂.

2. The nitrogen balance experiments seem rather inconclusive—two experimental rats stored less nitrogen, while one deposited more than the respective controls—and a comparison of 15 pairs of nitrogen estimations shows that the experimental rats were favoured in 6, and the controls in 9, a result easily obtained by chance. The controls however stored on the whole decidedly more nitrogen than the experimental animals. When the biological value of the ingested nitrogen is calculated according to the method of Mitchell a slightly better efficiency is found in the case of the controls, 12 results being in favour of the latter, 3 even, and none in favour of the experimental rats—a result hardly due to chance.

3. There is a strong indication that the gains in weight made by the two sets of rats are of different composition.

4. To this may be added the evidence brought forward in the earlier paper [Kon, 1929] that the carbon/nitrogen ratio is slightly increased in the urine of experimental rats deprived of vitamin B₂, as compared with individual controls.

Is it possible to derive from these observations any conclusions concerning the rôle of vitamin B₂ in metabolic functions? In an effort to answer this question the points enumerated above will be subjected to a more detailed analysis.

(1) The phenomenon described under 1 is not characteristic for vitamin B₂ deficiency. It has been found by other investigators, in well controlled experiments, in the case of various deficiencies and is therefore not pathognomonic of the lack of vitamin B₂. Anderson and Smith [1924] in their study by the individual control method of the effect of acute scurvy on the subsequent nutrition and growth of guinea pigs came to the conclusion that "there are factors resulting in loss of body weight which operate in addition to fasting, and which depend on scurvy itself. In other words, inanition alone does not cause as severe a loss as when accompanied by lack of vitamin C." Mitchell and Carman [1926, 1] have shown by means of the same method that a ration composed mainly of maize, when supplemented with NaCl, induced 50 % faster gains in weight of both rats and chickens than the saltless ration. The authors remark that "the growth data of this experiment afford a striking demonstration of the fact that the utilisation of food energy by growing animals may be greatly impaired by an improper balance among indispensable dietary factors." Similar differences are reported by Mitchell and Beadles [1930] and Beadles, Braman and Mitchell [1930] in the case of diets deficient in cystine and by Jackson [1929] in a deficiency of tryptophan, while Burr and Burr [1930] made observations of the same nature in their study of the fat deficiency disease (individual controls were not employed in this study).

The estimation of the "physiological efficiency" of various proteins, as carried out by the earlier method of Osborne and Mendel [1916], is nothing else than the comparison of food mixtures based on a similar "individual control" method: the rats (all males) were of approximately the same body weight at the beginning of the experiment and were offered daily limited amounts of food "precisely alike in energy content, inorganic and accessory food ingredients, etc." and differing only with regard to the proteins under investigation. The rats made widely divergent weight gains while consuming equal amounts of these rations. On commenting upon these excellent experiments Mitchell [1924, 3] very aptly remarks "these experiments suggest an interesting relation, hitherto uninvestigated as far as the author is aware, between the balance of nutrients in a ration and the utilisation of its energy."

We are here confronting a general phenomenon, namely the growth-limiting influence of the lack or insufficiency of any indispensable food constituent manifesting itself in an impaired utilisation of the food.

For a certain time some vitamins were referred to as "growth-promoting," but there is certainly no doubt that every essential food constituent is growth-promoting in the sense that its absence from the diet will sooner or later cause stoppage of growth. It seems that sufficient evidence was cited above to prove that, as a general rule, one of the ways in which this growth-inhibiting action will come into operation is through an impaired utilisation of the ingested energy, and that in this respect all indispensable nutrients, vitamins, minerals, or amino-acids, manifest a great similarity of action.

A deficiency of an essential ingredient of the diet may or may not have

a depressing influence on the appetite [Beadles, Braman and Mitchell, 1930], at least in the earlier periods of the deficiency. Ultimately probably the general derangement will be sufficiently pronounced to cause in every case a diminished food intake.

The most conspicuous association of an essential food constituent with appetite is certainly to be found in the case of the vitamin B complex and of vitamin B₁. The inanition sets in here so rapidly and is of such severity that no difference in the rate of the loss of weight can be found between "experimental" animals and controls, when the individual control method is used [Kon and Drummond, 1927; Kon, 1929].

(2) Passing now to the second point of interest relating to the study of vitamin B₂ deficiency as presented in this paper, namely to the nitrogen balance experiments, it seems that a less favourable balance would naturally be expected in the case of the experimental rats, having consumed the same amount of, but stored less, energy than the controls, and that therefore the results on the whole agree with the expectation. It was shown by Mitchell and Carman [1926, 1] that more favourable nitrogen balances were found for every one of their control rats and chicks receiving an addition of NaCl, as compared with the experimental ones. The evidence available does not seem to show that vitamin B₂ is any more connected with the metabolism of nitrogen than is sodium chloride.

(3) The question of the composition of the tissues added by the two sets of rats in the present experiment is certainly a very pertinent one. Little more can be said on this point than that the mere estimation of differences in weight does not yield sufficient information, and that in studies of this type, that is to say in the investigation of the relation of any indispensable nutrient to the metabolism of matter and energy, the actual chemical make-up and energy content of the added weight should be estimated as a matter of routine.

(4) Reviewing in turn the value of the finding reported in the previous paper [Kon, 1929], that in the absence of vitamin B₂ there is an increased excretion of carbon compounds as compared with nitrogenous bodies in the urine, it is necessary to ask how significant such an observation is. An alteration of the urinary C/N ratio in scorbutic guinea pigs has been reported by Jarussowa [1928], and Roche [1930] states that the ratio is increased in the terminal stage of vitamin B deficiency. Bickel [1924, 1930] is of the opinion that this symptom is present in disturbances of metabolism of a non-avitaminous character. Laclau and Marenzi [1930] report that the C/N ratio is increased in the urine of rats on a diet poor in cystine as compared with normally fed rats. Shipp and Zilva [1928] have criticised the experiments of Jarussowa and doubt whether the development of scurvy is associated with an increased C/N ratio in the urine. They admit however that their experiments are inconclusive in view of the discordant results obtained (there was a definite increase in the ratio in one of their guinea pigs while no such deviation was observed in the case of the other). While the question can be definitely solved

only by means of suitably controlled experiments (with equalised food intake for control and experimental animals), it seems at least doubtful at present whether the alteration of the urinary C/N ratio is characteristic for vitamin B₂ deficiency. It would be best to abstain from far-reaching conclusions, and in particular the author is of the opinion that, while the importance of vitamin B₂ for normal economy is self-evident, there are no sufficient grounds for making it responsible for any particular branch of metabolism.

In conclusion it may be said that while vitamin B₂ is undoubtedly in some way linked with the metabolic processes of the body, it is not at all evident whether this connection is to any extent of a different type from that of the general dependence of normal metabolic exchange upon the availability of any one of the indispensable food ingredients.

SUMMARY.

1. A study by the individual control method of the nitrogen balance in vitamin B₂ deficiency in the rat is reported.
2. The relation of vitamin B₂ to metabolism is discussed.

Note added, April 8th. Still and Koch (1928) have measured, by Mitchell's method, the biological values of diets containing one half of the nitrogen from yeast and one half from caseinogen (total N 2.9 %) and found no supplementary relation between the two proteins.

REFERENCES.

- Anderson and Smith (1924). *J. Biol. Chem.* **61**, 181.
 Beadles, Braman and Mitchell (1930). *J. Biol. Chem.* **88**, 615.
 Bickel (1924). *Munch. med. Woch.* **71**, 1603.
 — (1930). *Med. Welt*, No. 19.
 Burr and Burr (1930). *J. Biol. Chem.* **86**, 587.
 Hunt (1928). *J. Biol. Chem.* **79**, 723.
 Jackson (1929). *J. Biol. Chem.* **84**, 1.
 Jarussowa (1928). *Biochem. Z.* **198**, 128.
 Kennedy and Palmer (1928). *J. Biol. Chem.* **76**, 591.
 Kon (1928). *Biochem. J.* **22**, 261.
 — (1929). *J. Nutrition*, **1**, 467.
 — and Drummond (1927). *Biochem. J.* **21**, 632.
 Laclau and Marenzi (1930). *Senana Med.* **37**, 1208.
 Mitchell (1924, 1). *J. Biol. Chem.* **58**, 873.
 — (1924, 2). *J. Biol. Chem.* **58**, 905.
 — (1924, 3). *Physiol. Rev.* **4**, 424.
 — and Beadles (1930). *J. Nutrition*, **2**, 225.
 — Beadles and Kruger (1927). *J. Biol. Chem.* **73**, 767.
 — and Carman (1926, 1). *J. Biol. Chem.* **68**, 165.
 — (1926, 2). *J. Biol. Chem.* **68**, 183.
 — and Hamilton (1929). The biochemistry of the amino-acids.
 (The Chemical Catalog Co., New York.)
 Osborne and Mendel (1916). *J. Biol. Chem.* **26**, 1.
 — Mendel and Ferry (1919). *J. Biol. Chem.* **37**, 223.
 — and Wakeman (1919). *J. Biol. Chem.* **40**, 383.
 Roche (1930). *Bull. Soc. Chim. Biol.* **12**, 342.
 Shipp and Zilva (1928). *Biochem. J.* **22**, 1449.
 Steenbock (1923). *Science*, **58**, 449.
 Still and Koch (1928). *Amer. J. Physiol.* **87**, 225.
 Sure (1928). *J. Biol. Chem.* **76**, 673.

LX. THE BIOCHEMISTRY OF DRY-ROT IN WOOD.

II. AN INVESTIGATION OF THE PRODUCTS OF DECAY OF SPRUCE WOOD ROTTED BY *MERULIUS LACHRYMANS*.

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It was shown by us in a previous communication [1929] that, contrary to the usually accepted view, the action of *Merulius lachrymans* (the fungus responsible for dry-rot) on wood was to destroy the major part of the cellulose fraction of the lignocellulose complex and to leave the lignin component unaffected. It was found that the so-called hemicelluloses, which are present in woody tissues, were also resistant to fungal attack, whereas the hexosans, mannan and galactan were completely destroyed.

We have been able to confirm our previous observations as to the general action of the fungus on wood. The present investigation is concerned with the detailed analysis of the nature of the various fractions that can be isolated from rotted wood, together with a comparison of fractions isolated in a similar manner from sound wood.

EXPERIMENTAL.

The material employed was a 5th grade Norwegian spruce wood that had been rotted by *M. lachrymans*, and the various fractions were isolated according to the details given by Dore [1920]. The whole of the wood used (both sound and rotted material) came from the same source, but it was found that the decayed wood was decomposed to a very much greater extent than the sample used in our previous investigation. On this account a quantitative analysis was repeated, and the results are given below.

Compared with our previous sample, the rotted wood used in this investigation shows a considerable decrease in the alkali-soluble fraction (19.10 % compared with 30.0 %). A decrease is also shown in the lignin content (40.90 % compared with 43.00 %) as well as in the value for cellulose (9.88 % compared with 11.90 %), whereas, on the other hand, the alcohol-soluble and water-soluble fractions both show a marked rise over the percentages previously recorded.

Table I.

Treatment				Sound wood	Decayed wood
				% *	% *
Extracted by benzene	1.70	1.48
" alcohol	1.60	19.80
" water	2.50	8.00
" 5 % NaOH in the cold	3.80	19.10
Cellulose	55.00	9.88
Lignin	28.00	40.90
Mannan	7.60	0.00
Galactan	0.10	0.00
Total	100.30	99.16
Ash	0.86	0.61
Natural moisture	10.00	17.30

* Calculated on ash-free material dried at 100°.

The isolation of the various fractions described below was carried out on a large scale. The sample of sound wood was reduced to sawdust and the rotted material was ground to a fine powder in a mortar. The wood (both sound and decayed) was then treated in turn with the reagents described in Table I (*i.e.* benzene, alcohol, water and 5 % NaOH), and the cellulose was isolated after chlorination of the extracted samples and removal of the lignin either with sodium sulphite or with alcohol. Each particular sample was then worked up separately, and the various products were isolated and examined.

It will be convenient to detail the nature of the fractions obtained from the rotted and sound material separately and to discuss the theoretical implications later.

Fractions isolated from rotted wood.

Benzene-soluble fraction. After extraction of the decayed material with benzene and distillation of the solvent, the benzene-soluble fraction was obtained as a viscous, rather mobile, brown mass. This was heated on the water-bath with *N*/10 alcoholic caustic potash for 1 hour and the contents of the flask were then acidified with hydrochloric acid. A heavy brown precipitate separated, which on treatment with ether partially dissolved leaving a brown residue. The residue readily reduced Fehling's solution and formed an oxime. The ethereal extract was distilled under reduced pressure; the residue was taken up with a small amount of *N*/10 sodium hydroxide solution, and the sodium salt of a fatty acid was salted out from the alkaline solution with sodium chloride. Unfortunately the amount of fatty acid obtained was too small to allow of identification. The solution, after the initial precipitation of fatty acid and resin, was distilled under reduced pressure and tested with sulphuric acid and β -naphthylamine for glycerol; a positive reaction was obtained.

The benzene-soluble fraction of rotted wood is therefore composed of a fat or fats and two resins, one of which is soluble and the other insoluble in ether.

Alcohol-soluble fraction. On evaporation of the solvent, there remained a brown, amorphous mass, which partially melted at 60°. It was odourless and

soluble in acetone, glacial acetic acid and sodium hydroxide solution and insoluble in benzene and light petroleum. It was precipitated from glacial acetic acid solution by the addition of water or from sodium hydroxide solution by the addition of hydrochloric acid. It was purified for combustion by dissolving in glacial acetic acid and precipitating with water. The precipitated product was washed and dried *in vacuo*. Analysis indicated the empirical formula, $C_{22}H_{25}O_8$. (Found: C, 63.05 %; H, 6.11 %; $C_{22}H_{25}O_8$ requires C, 63.3 %; H, 5.99 %.) The substance reduced Fehling's solution and the presence of an aldehydic group was confirmed by the preparation of an oxime. Determination of the molecular weight showed that the above formula should be doubled. The substance is therefore not of the nature of the metalignin isolated by Dorée and Barton-Wright [1927], as was previously suggested by us. The substance contained two methoxyl groups ($OMe = 9.21\%$) and yielded a tetrabenzoyl derivative showing the presence of four hydroxyl groups. (Found: C, 69.0 %; H, 5.6 %; $C_{44}H_{46}O_{12}$ ($O.CO.C_6H_5$)₄ requires C, 69.1 %; H, 5.28 %.) All attempts to separate the material into different fractions failed and from its reactions it appeared to be perfectly homogeneous. Its extended formula can therefore be written $(CH_3O)_2.C_{41}H_{39}O_9(OH)_4.CHO$.

Water-soluble fraction. The water-soluble fraction was acid to litmus and when evaporated to dryness under reduced pressure yielded a dark-brown, viscous mass, which rapidly reduced Fehling's solution. Neither tannins nor organic acids were found to be present, and the nature of the acidity remained undetermined.

The extract was decolorised by boiling with animal charcoal, and methyl alcohol was added to the filtrate, when a copious white precipitate separated. This was found to be entirely composed of glucose (identified by osazone and Bertrand's test). This, with the exception of a trace of acid the nature of which we were unable to determine, was the sole constituent of the water-soluble fraction.

5 % sodium hydroxide-soluble fraction. When the rotted wood, after previous extraction with benzene, alcohol, and water was treated for 36 hours in the cold with a 5 % solution of sodium hydroxide, a dark-brown liquid was obtained. This was filtered off from the residue and together with the first washing was treated with an excess of acetic acid, which produced a flocculent brown precipitate. After standing for 24 hours, the supernatant liquid was poured off, and the precipitate was washed several times with water to free it from acid. It was air-dried for several days and finally dried *in vacuo*. During the course of drying it became very much darker in colour.

The supernatant liquid, which was of a pale straw colour, was dialysed for 8 days to free it from acetic acid and sodium acetate and then evaporated to small bulk under reduced pressure and treated with an equal volume of alcohol. The small amount of dark-brown precipitate was filtered off, washed with alcohol and dried *in vacuo*. The filtrate was evaporated to dryness, when a dark-brown evil-smelling mass was left as a residue.

The alkali-soluble fractions of wood contain the so-called "hemicelluloses," bodies which are composed of hexose and pentose sugars as well as uronic acids of the galacturonic and glycuronic type. A number of investigations have recently appeared on these compounds. O'Dwyer [1926, 1928], for example, was able to isolate two such bodies from beech wood sawdust, to which she gave the names of hemicellulose A and hemicellulose B respectively. Hemicellulose A was insoluble in water, but soluble in dilute solutions of sodium hydroxide, from which it could be precipitated by acids; hemicellulose B was soluble in water, but insoluble in alcohol. The two substances which we have been able to isolate from decayed spruce wood appear to be of the nature of hemicelluloses, but owing to the fact that they showed a number of differences from the products isolated by O'Dwyer, as well as from the corresponding fractions isolated from sound spruce wood, we prefer to name them hemicellulose A₁ and hemicellulose B₁.

Hemicellulose A₁. This was a dark-brown amorphous body, insoluble in water, but slightly soluble in alcohol. It was readily soluble in weak solutions of sodium hydroxide and could be reprecipitated from alkaline solution by the addition of acid. It reduced ammoniacal silver nitrate but not Fehling's solution.

20 g. of the material was hydrolysed with 200 cc. of 3 % sulphuric acid for 6 hours. At the end of this time the residue was filtered off, and the requisite amount of barium hydroxide was added to neutralise the sulphuric acid. The precipitate of barium sulphate was filtered off, and the filtrate was found to be still slightly acid. A further quantity of barium hydroxide was added until the solution was alkaline, and the precipitate was filtered off and preserved. The filtrate was evaporated under reduced pressure to a thin syrup and extracted several times with absolute alcohol and then with 80 % alcohol.

The two alcoholic extracts were tested for mannose as the hydrazone, arabinose as the osazone, xylose as the cadmium xylobromide salt, galactose as mucic acid and glucose as the osazone. The portion soluble in absolute alcohol gave the characteristic crystals of cadmium xylobromide and showed the presence of xylose in the complex. No other sugar was found to be present in this portion. The extract obtained with 80 % alcohol gave galactosazone melting at 189-190°. The presence of galactose was confirmed by the preparation of mucic acid.

The percentage of uronic acid in the complex, estimated by the method of Nanji, Paton and Ling [1925], was 10.73 %. The second barium precipitate mentioned above was treated with the calculated quantity of sulphuric acid to decompose the barium salt of the uronic acid. The barium sulphate was filtered off, and the filtrate was saturated with cinchonine at 100°. On cooling, large crystals of cinchonine sulphate separated out, owing to the presence of a slight excess of sulphuric acid in the mixture; at the same time, a mass of fine needle-shaped crystals was also obtained. The fine needles were centrifuged off, freed from cinchonine sulphate by washing with alcohol and twice

recrystallised from water (M.P. 211–214°). We have been unable to find in the literature a uronic acid the cinchonine salt of which melts at this temperature. Up to the present time, the only uronic acids that have been isolated from hemicelluloses are galacturonic and glycuronic acids. It is possible that we are here dealing with a penturonic acid, that derived from arabinose or xylose. Gymnospermous woods have not been investigated with regard to the nature of their hemicellulose content, and we are at present engaged in making a fuller examination of this point, as well as in preparing pure samples of the uronic acids from xylose and arabinose for purposes of comparison.

When treated with fuming hydrochloric acid (sp. gr. 1.20) hemicellulose A_1 after 20 hours yielded a lignin-like body having OMe 12.4 %. The original sample before acid treatment had OMe 6.68 %.

The nature and amounts of the products obtained from hemicellulose A_1 are shown in Table II. An attempt was made to determine the nature of the

Table II.

	%
Xylose	1.17
Uronic acid (nature unknown)	10.73
OMe of hemicellulose A_1 before treatment with HCl	6.68
OMe of residue after treatment with HCl (<i>sp. gr.</i> 1.2)	12.40
OMe of residue calculated on original material ...	6.15
OMe hydrolysed by HCl	0.53
Residue insoluble in HCl	49.64

linkages between the component parts of the molecule. Portions of 5 g. of the hemicellulose A_1 were hydrolysed with 3 % sulphuric acid for 1, 3, 5 and 10 hours respectively and the methoxyl content, furfuraldehyde and carbon dioxide values were determined. The figures, calculated as a percentage of the original material, are given in Table III. It is clear that the linkages between

Table III. *Effect of acid hydrolysis for varying periods on hemicellulose A_1 .*

Fraction	Original material %	1 hour %	3 hours %	5 hours %	10 hours %
Xylose	1.17	0.00	0.00	0.00	0.00
Uronic acid	10.73	4.10	4.40	4.40	4.89
Methoxyl	6.68	6.52	6.45	6.30	6.00

the component parts of the complex differ from one another. Part of the methoxyl is hydrolysable by dilute acid, and the ease of hydrolysis suggests that the linkage is of an ester type, while the remainder of the methoxyl is attached to the portion insoluble in fuming hydrochloric acid, *i.e.* the lignin-like fraction, and the linkage here is of a more resistant nature, probably of an ether type.

The uronic anhydride content exhibited a peculiar feature, namely, a large initial decrease (7 %) after hydrolysis for one hour, but showed an increase during the remaining period. This result again suggests that there is more

than one type of linkage present in the complex. During the first hour, a weak linkage is destroyed, but during the remaining period over which hydrolysis is continued, a more resistant linkage is attacked, and one which prevents complete decarboxylation from taking place when uronic acids are estimated by the method of Nanji. Paton and Ling. The mild acid hydrolysis breaks this linkage slowly, and the yield of carbon dioxide recorded on decarboxylation represents the sum of the yields of gas from the acid set free by the mild acid hydrolysis and that set free by 12 % hydrochloric acid in the course of estimation. In order to nullify the effect of the latter hydrolysis, the time during which the estimations were carried out was kept constant. In this way the effect of the mild hydrolysis became apparent.

The fraction of rotted wood soluble in sodium hydroxide solution can therefore be divided into two portions, a part insoluble in fuming hydrochloric acid, which contains methoxyl groupings, but yields no carbon dioxide or furfuraldehyde when treated with hydrochloric acid, and may well be a body of lignin-like nature; and a second part composed of a mixture of pentose (xylose) hexose (galactose) and an unknown uronic acid. These constituents may be removed separately from the complex, together with a certain amount of methoxyl residues. The linkages between the xylose, galactose and uronic acid have not been completely defined by the methods employed, but it is evident that the pentose is more loosely combined in the complex than galactose and that the uronic acid is linked in such a manner that it is shielded from decarboxylation. This might be achieved by esterification or by the linkage of one of the other components of the complex to one of the carbon atoms in the uronic acid molecule so that a protective action is exercised over the carboxyl group. The possibility of esterification preventing decarboxylation is remote. Until we have carried out further work on the point, the first alternative will have to stand.

Hemicellulose B₁. Hemicellulose B₁, isolated in the manner described above, was a dark-brown powder, readily soluble in water. Unfortunately the amount available was too small to allow of any extensive observations being made on its properties. It contained 7.12 % of pentose; the methoxyl content was 3.19 % and the uronic acid value 7.68 %. When treated with hydrochloric acid (sp. gr. 1.2) for 24 hours it gave an insoluble residue of 31.88 %. It thus appears that here, as in the case of hemicellulose A₁, the true hemicellulose portion of the molecule is linked with a lignin-like fraction.

Cellulose fraction. The cellulose was isolated from decayed wood by chlorination after previous extraction in the cold with 5 % sodium hydroxide and subsequent removal of the chlorolignin by repeated extraction with boiling alcohol. It could also be isolated by boiling the chlorinated product with sodium sulphite. Both methods gave similar results. The residue, after extraction of the lignin with alcohol, was air-dried, when it was obtained as a hard dry mass, which could be reduced to a fine powder by grinding in a mill. It showed little fibrous structure. Small portions of the material were treated

with calcium hypochlorite, but in no case was any bleaching action observed. Uronic anhydride amounted to 3.23 % and pentose to 0.39 %. The physical nature of the material, together with the increased uronic acid value compared with cellulose isolated from normal wood (see below), suggested that the cellulose had suffered oxidation under fungal action; tests for oxycellulose were therefore carried out. The material rapidly reduced Fehling's solution and showed a marked affinity for basic dyes. With α -naphthol and sulphuric acid it developed a violet colour and with thymol and sulphuric acid a rose tint. Subjection to the Cannizzaro reaction abolished the reducing properties. The residue after treatment with strong sodium hydroxide solution was filtered and washed free from alkali and then tested with Fehling's solution; no reduction occurred. The filtrate, when acidified with hydrochloric acid, yielded a white precipitate which also failed to reduce Fehling's solution. The filtrate was neutralised and tested with Fehling's solution, but showed no reducing properties. Presumably an aldehydic grouping present in the cellulose of the decayed wood had suffered simultaneous oxidation and reduction under the influence of the Cannizzaro reaction, giving rise to an insoluble alcohol and the sodium salt of an insoluble acid. These tests indicate that part at least of the cellulose contained in rotted wood consists of oxycellulose. Treatment with 17.5 % sodium hydroxide solution according to the method of Schorger gave a yield of 22.9 % of α -cellulose. No attempt was made to estimate either β - or γ -cellulose.

Lignin fraction. The alcoholic solution obtained from the extraction of chlorolignin was distilled under reduced pressure until the chlorolignin began to be deposited as a hard brown mass on the bottom of the flask. This was removed, ground to a fine powder and washed with a small amount of water until free from sodium chloride (which remained from an earlier brine treatment of the chlorinated wood).

The chlorolignin thus isolated could be separated into two fractions: one soluble in glacial acetic acid and one soluble in sodium hydroxide solution. The chlorolignin was first treated with glacial acetic acid in the cold or occasionally with gentle warming, until after repeated extraction no more would pass into solution. The residue was washed free from acid and treated with a 1 % solution of sodium hydroxide. The acid fraction was isolated by pouring into a large volume of water, when a flocculent brown precipitate settled out. This was filtered off, washed, and dried *in vacuo*. The portion soluble in dilute sodium hydroxide solution was precipitated by the addition of dilute hydrochloric acid, washed free from acid and dried *in vacuo*.

Glacial acetic acid fraction. Further purification for combustion was effected by re-dissolving in glacial acetic acid and again precipitating with water. (Found: C, 47.6, 47.4 %; H, 3.72, 3.6 %; Cl, 19.22, 19.07 %.) No formula can be constructed from these figures and the acetic acid-soluble fraction is evidently a mixture. Further evidence that a mixture had been obtained can be seen from the figures found for the combustion of the benzoyl derivative,

in which the percentages for hydrogen are higher than for the original product. (C, 54.64, 54.22 %; H, 4.48, 4.33 %; Cl, 11.91, 11.61 %.)

Sodium hydroxide-soluble fraction. This also proved to be a mixture and the figures found for carbon on combustion showed an even greater variation than those obtained for the acetic acid-soluble fractions. (C, 43.05, 43.15, 48.1, 48.9 %; H, 4.09, 3.85, 3.68, 4.07 %; Cl, 15.87, 15.30 %.)

Fractions isolated from sound spruce wood.

For the purpose of comparison, fractions were now isolated from sound spruce wood in exactly the same manner as from the decayed material. The wood was reduced to sawdust by running the end of a block against a fast-running circular saw. In this way 1400 g. of material were prepared. This sawdust was reduced to wood flour of the same grade as the rotted sample by grinding in a mill and was extracted successively with benzene, alcohol, water and 5 % sodium hydroxide solution in the cold. The cellulose was isolated by chlorination of the wood after alkaline extraction followed by boiling with alcohol or sodium sulphite.

Benzene-soluble fraction. On evaporation of the solvent, a practically colourless, odourless, slightly viscous resinous mass remained. This was boiled for one hour with *N*/10 alcoholic caustic potash; the contents of the flask were then acidified with hydrochloric acid and the precipitate of fatty acids and resins filtered off. On extraction with ether, the resins entirely passed into solution, together with a portion of the fatty acids. The ethereal extract was evaporated to small bulk, and the residue was dissolved in a small amount of sodium hydroxide solution, from which the fatty acids were salted out, leaving the resins in solution. The filtrate, after initial acidification with hydrochloric acid, was distilled under reduced pressure and tested for glycerol with β -naphthylamine and sulphuric acid: a positive reaction was obtained.

The benzene-soluble fraction isolated from sound wood, therefore, like that of the rotted material, is composed of fats and resins.

Alcohol-soluble fraction. After removal of the alcohol by distillation under reduced pressure, a small amount of a dark-brown, oily liquid remained. This was found to be insoluble in glacial acetic acid and was too small in amount for identification.

Water-soluble fraction. On evaporation of the aqueous extract under reduced pressure, a dark-brown, slightly viscous mass remained. This reduced Fehling's solution and tests with methylene blue showed it to be composed of tannins and reducing sugars in small amount.

Sodium hydroxide-soluble fraction. The residue of the sound wood after extraction with benzene, alcohol and water was allowed to stand in a 5 % solution of sodium hydroxide for 36 hours in the cold. At the end of this period the residue was filtered off yielding a golden-brown solution. The first washings from the residue were added to the filtrate and the whole was acidified with acetic acid. A small amount of a light-coloured precipitate

separated out. The supernatant liquid was removed and the precipitate was washed free from acid and dried *in vacuo*. Unlike the corresponding precipitate from decayed wood, it underwent no darkening in colour on drying. The filtrate, after acidification, was dialysed for 8 days to remove excess of acetic acid and sodium acetate and was then evaporated to small bulk under reduced pressure. When alcohol was added, a slight precipitate was formed, but this was too small in amount to collect.

The first precipitate which separated on acidification of the alkaline extract presumably corresponds with the hemicellulose A of O'Dwyer and the second, which formed on the addition of alcohol, to her hemicellulose B. In any case her nomenclature will be followed here for the sake of convenience and to save confusion.

Hemicellulose A. The fawn-coloured precipitate formed on the addition of acid to the alkaline extract of sound wood was treated with fuming hydrochloric acid (sp. gr. 1.2) for 24 hours and left a residue of 47.7 %, which had OMe 12.72 %. The original material had OMe 7.87 %, and contained 3.61 % of uronic anhydride and 5.94 % of pentose.

12 g. of the material were hydrolysed with 3 % sulphuric acid for 5 hours at 100°. At the end of this time, the residue was filtered off, washed and dried and amounted to 55.9 % of the original material. The filtrate was neutralised with barium hydroxide in exactly the same way as for the fraction isolated from decayed wood, and the precipitate of barium sulphate was removed and preserved. The residue of the neutralised filtrate after evaporation was extracted first with absolute alcohol and then with 80 % alcohol, and the two extracts were tested for mannose, galactose, arabinose and xylose. The absolute alcoholic extract was found to contain xylose and the 80 % alcoholic extract galactose, while the barium sulphate precipitate contained the unknown uronic acid described above for hemicellulose A₁. The constitution of hemicellulose A is summarised in Table IV. Owing to the small amount of

Table IV.

	%
Xylose	5.90
Uronic acid	3.61
OMe	7.89
Residue after treatment with HCl (<i>sp. gr.</i> 1.2) ...	47.70
OMe in residue after treatment with HCl	12.72
OMe calculated on original material	6.07
OMe readily hydrolysed by HCl $\left(7.89 - \frac{12.72 \times 47.7}{100}\right)$	1.82

material available it was impossible to carry out a series of hydrolyses over varying periods of time, as in the case of hemicellulose A₁. We have, on this account, no evidence as to the nature of the linkages between the component parts of the complex.

Cellulose. The cellulose was isolated after alkaline extraction of the sound wood and chlorination, by dissolving out the chlorolignin either with alcohol

or with alkaline sodium sulphite. The percentages obtained were the same in both cases; 55.0 % as compared with 9.88 % in rotted wood.

The cellulose was a white fibrous substance, which did not reduce Fehling's solution and showed none of the properties of oxycellulose. It yielded 80.65 % of α -cellulose and 8.97 % of pentosan, calculated from the furfuraldehyde precipitated as phloroglucide, after making allowance for the uronic acid content. The percentage of uronic acid was 0.95 %.

Lignin fraction. The chlorolignin was isolated from the sound wood in the same manner as described above for decayed material. Separation into two fractions was again effected by means of glacial acetic acid and 1 % sodium hydroxide solution. On combustion the samples gave the following values:

Glacial acetic acid fraction: C. 47.89, 48.77, 52.9, 42.1, 43.1 %; H, 4.54, 4.94, 4.79, 4.66, 5.42 %; Cl. 18.77 %; OMe. 4.7 %.

Sodium hydroxide-soluble fraction: C. 49.53, 53.14, 50.77 %; H, 4.03, 4.87, 4.77 %; Cl. 12.93, 12.83 %.

From the wide variations in these figures, it is clear that, as in the case of the chlorinated products isolated from decayed wood, we are dealing with mixtures and not homogeneous products. The main difference lies in the fact that the samples isolated from fungus-infected wood possess a higher percentage of oxygen than the corresponding samples from sound material.

DISCUSSION.

It is necessary to emphasise the fact that throughout the course of this investigation only completely decomposed material was employed. We are, on this account, unable to advance any views as to the exact course of fungal metabolism at each stage in the progress of decay. It is clear, however, that the presence of the fungus in the wood produces far-reaching and profound changes in the wood components, especially in the two most important fractions of the lignocellulose complex, namely, the cellulose and lignin.

The fact that the cellulose has fallen by 81 % in the rotted material and that the residue remaining is much altered in nature, indicates that the chief action of the mycelium is exercised on this particular component of the woody tissues. Two reactions appear to have progressed simultaneously here: a hydrolysis of the cellulose to glucose and an oxidation to oxycellulose. Presumably the comparatively large amount of glucose located in the water-soluble fraction of the rotted wood was originally derived from the cellulose and was left as an unconsumed residue by the fungal mycelium in its passage through the wood. In any case, it is the α -cellulose fraction of the total cellulose of the wood that is chiefly affected by the presence of the fungus, as there is a fall from 47.6 % to 2.26 % in α -cellulose, calculated on the total wood.

It was shown by us in our previous communication that the hemicellulose fractions of the wood were apparently unaffected by fungal infection; although at the time we were unable to account for the increased solubility of decayed

wood in sodium hydroxide solution. It is very unfortunate in this connection that different workers have defined the term hemicellulose in different ways. Candlin and Schryver [1928], for example, have introduced the term "polyuronide" to include substances containing linked uronic acids and sugars, whether the latter be pentoses or hexoses. Such a definition includes pectins as well as hemicelluloses of the type described by O'Dwyer and gums of the gum arabic type recently described by Norman [1929]. More recently, Norris and Preece [1930] have agreed with the classification put forward by Candlin and Schryver and have further suggested that the term hemicellulose should be restricted to bodies which give no uronic acids but only sugars on hydrolysis. It appears to us superfluous to introduce the term polyuronide in this connection, since it brings into the same class such diverse bodies as the pectins and hemicelluloses isolated by O'Dwyer, while the terms hexosan and pentosan still appear to be quite adequate to describe compounds only containing hexoses or pentoses, and some synthetic name may be derived for products consisting of mixtures of these two classes of sugars. The term hemicellulose is used here for products soluble in dilute sodium hydroxide solution, but insoluble in sodium carbonate solution, which on hydrolysis yield hexose and pentose sugars as well as uronic acids. Using such a definition, it will be seen that the hemicellulose products of wood are practically unaffected by *M. lachrymans*. In both sound and rotted wood, the same products are obtained on hydrolysis, namely, xylose, galactose and an unknown uronic acid and in much the same proportions. A residue insoluble in fuming hydrochloric acid is also found in both cases and it is curious that no mention is made by either O'Dwyer or Norman that a residue remains when this material is hydrolysed by weak acid. The fact that a residue of nearly 50 % remains after treatment with fuming hydrochloric acid for 24 hours considerably invalidates the Willstätter and Zechmeister [1913] method of estimating lignin in woody tissues.

It is peculiar that the fungus fails to remove the hemicellulose fraction of the wood, since the hemicelluloses contain both hexose and pentose sugar in their composition. On the other hand, *M. lachrymans* is markedly sensitive to the action of acids and even such weak acids as tannic and gallic acid inhibit its growth and produce a toxic effect. Since uronic acids are produced by the hydrolysis of hemicelluloses, it may well be on this account that the fungus fails to attack the hemicellulose fraction.

Campbell and Booth [1929] have investigated the action of *Trametes serialis* on Sitka spruce and like other investigators have observed an increase in the solubility of the rotted material in dilute solutions of caustic alkalis, as well as a decrease in cellulose content. From the sum of the percentages of the fractions in the rotted wood, they have put forward the suggestion that a substance is formed, which, while insoluble in water, is not estimated as either cellulose, lignin or pentosan and is possibly of the nature of a carbohydrate degradation product. From a comparison of the effect of the fungus

on sawdust with the action of an aqueous acid hydrolysis at 100° they have formulated the view that, in the earlier stages at least, the effect of infection of woody tissues by a "brown-rot" type of fungus is to be regarded as strictly comparable with aqueous acid hydrolysis and consider that support for this suggestion is to be found in the acid nature of rotted wood. In the absence of any experimental work on the nature of the alkali-soluble fraction of rotted material, it appears somewhat premature to advance any theory to account for the effects of fungi of the "brown-rot" type. In the later stages of decay, our results indicate that oxidation plays a very much more prominent part in the process than anything akin to an aqueous acid hydrolysis.

Although the total lignin content of wood is unaffected by *M. lachrymans*, nevertheless, the fungus produces a number of changes in this component of the lignocellulose complex. One curious feature of the problem is the occurrence of a definite compound of composition $C_{44}H_{50}O_{16}$ in the alcohol-soluble fraction of decayed wood. This substance shows the reactions ordinarily associated with an "alkali" lignin and very closely approximates in composition to the lignin isolated by Powell and Whittaker [1924, 1925] from flax by treatment of the fibres with 8-12 % solutions of sodium hydroxide under pressure for several hours. We had hoped that this product would have been a substance of simpler composition and possibly of the nature of "metalignin" isolated by Dorée and Barton-Wright [1927], which possessed the molecular composition, $C_{20}H_{20}O_6$.

It is difficult to account for the presence of this lignin in the alcohol-soluble fraction. It was the only homogeneous lignin product we were able to isolate throughout this investigation. The majority of investigators of the chemistry of lignin have assumed that the lignin fraction of the lignocellulose complex is a homogeneous body and numerous formulae have been put forward from time to time. From the results of our present work, this homogeneity does not appear to exist. The chlorolignins that were isolated, for example, from both sound and decayed wood were all mixtures. It is possible that lignin, using the name generically, is composed of closely related isomerides, perhaps differing only in methoxyl content, and, since they are colloidal in nature, it may be on this account that apparent homogeneity has been found, where in fact none existed. But whether lignin be a single substance or a mixture, it is highly susceptible to oxidation and is able to take up large amounts of oxygen [cf. Dorée and Hall, 1924; Dorée and Barton-Wright, 1929]. Apparently a similar result is brought about by the action of a "brown-rot" fungus on wood. The chlorolignins isolated from decayed wood all possessed a higher oxygen content than similar bodies isolated from sound material.

The action of *M. lachrymans* on wood is therefore to consume the hexosans, mannan and galactan, and then to attack the cellulose fraction of the complex. The latter is converted to glucose, the α -cellulose being primarily affected, while a small residue is left as oxycellulose. The hemicelluloses are not destroyed and the lignin fractions, though not removed, suffer oxidation.

SUMMARY.

1. A detailed investigation of the action of *Merulius lachrymans* (the fungus of dry-rot in wood) has been made on spruce wood. The action of the fungus is first to remove the easily hydrolysable fractions, mannan and galactan, and then to attack the cellulose. The latter is converted into glucose and consumed as such. At the same time a small residue of oxycellulose is left.

2. A homogeneous lignin body was extracted from the rotted wood with alcohol, which showed the molecular composition $C_{44}H_{50}O_{16}$ and possessed the extended formula $(OCH_3)_2 \cdot C_{41}H_{33}O_9(OH)_4 \cdot CHO$. From its reactions it appeared to be similar to the lignins which have been isolated from woody tissues by the action of caustic alkalis under pressure.

3. The hemicellulose fractions of spruce wood are unaffected by the fungus. An unknown uronic acid was isolated from the hemicellulose A fraction. The suggestion is put forward that this is a penturonic acid.

4. The total lignin content of the wood is practically unaltered, but marked oxidation has taken place, *i.e.* there is little or no delignification of the wood.

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REFERENCES.

- Barton-Wright and Boswell (1929). *Biochem. J.* **23**, 110.
Campbell and Booth (1929). *Biochem. J.* **23**, 566.
Candlin and Schryver (1928). *Proc. Roy. Soc. Lond.* B **103**, 365.
Dore (1920). *J. Ind. Eng. Chem.* **12**, 472, 476, 984.
Dorée and Barton-Wright (1927). *Biochem. J.* **21**, 290.
—— (1929). *J. Soc. Chem. Ind.* **48**, 9 T.
—— and Hall (1924). *J. Soc. Chem. Ind.* **43**, 257 T.
Nanji, Paton and Ling (1925). *J. Soc. Chem. Ind.* **44**, 253 T.
Norman (1929). *Biochem. J.* **23**, 1353, 1367.
Norris and Preece (1930). *Biochem. J.* **24**, 59.
O'Dwyer (1926). *Biochem. J.* **20**, 656.
—— (1928). *Biochem. J.* **22**, 381.
Powell and Whittaker (1924). *J. Chem. Soc.* **125**, 357.
—— (1925). *J. Chem. Soc.* **127**, 132.
Willstätter and Zechmeister (1913). *Ber. deutsch. chem. Ges.* **46**, 4201.

LXI. THE GLYCERIDE STRUCTURE OF BUTTER-FATS.

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THE glyceride structures of five butter-fats from cows fed under definite conditions have been investigated by isolation of sufficient of their fully-saturated glycerides to permit in each case of a detailed analysis of the fatty acids present in the latter by the ester-fractionation process. The component fatty acids of the whole butter-fats had already been determined [Hilditch and Sleightholme, 1930]. Combination of the analytical results for the whole fats with those for the fully-saturated glyceride portions furnishes much information as to the distribution of saturated and unsaturated acids among the glycerol molecules, and also throws some light on certain aspects of the distribution of the individual saturated acids. The objects of this communication are (i) to summarise the experimental data on the examination of the fully-saturated glycerides present in these five butter-fats and (ii) to discuss the general features of the glyceride structure of butter-fats revealed by these figures in conjunction with those formerly obtained for two New Zealand butter-fats [Hilditch and Jones, 1929] and, more recently, for an Indian cow ghee [Bhattacharya and Hilditch, 1931].

The various specimens will be referred to throughout this paper as follows.

- I. Shinfield, Berkshire; October 1928, cows partly on pasture, partly on winter diet.
 - II. Shinfield, Berkshire; March 1929, cows on winter diet with added coconut cake ration.
 - III. Shinfield, Berkshire; March 1929, cows on winter diet with added soya bean cake ration.
 - IV. Shinfield, Berkshire; May 1929, cows on early summer pasture.
 - V. Palmerston North, New Zealand; December 1928, cows on early summer pasture.
- A and B. New Zealand butter, market samples [Hilditch and Jones, 1929].
CG. Indian cow ghee from pasture-fed cows [Bhattacharya and Hilditch, 1931].

The butters I–IV were collected from members of the same herd of ten Shorthorn cows at the National Institute for Research in Dairying and V was

supplied by Dr F. H. McDowall from the Dairy Research Institute at Palmerston North; details of the feeding of the cows in question will be found in our former paper [1930, pp. 1099, 1100], and the general analytical characteristics of these five butter-fats and the data for the component fatty acids present in the whole fats have also been recorded [1930, pp. 1100, 1105, 1106].

Fully-saturated glyceride content of butter-fats I-V.

Determinations of fully-saturated glyceride content were carried out by the method which has been fully described in several recent papers [cf. Hilditch and Jones, 1929], the fat, in quantities of 100 g., being dissolved in acetone (1 litre) and oxidised by gradual addition of powdered potassium permanganate (400 g.) to the gently boiling solution. The only modification which has been made in the procedure as formerly described is that it has been found useful to have suspended in the solution clean dry silver sand (about 300 g.), which lessens the accumulation of a hard cake of mixed fat, oxidation products and manganese oxides, and facilitates its removal for subsequent reduction of the oxides of manganese by means of sodium bisulphite and dilute sulphuric acid. After separating as much of the acidic products of oxidation as possible by washing the ethereal solution with dilute aqueous ammonia, the crude fully-saturated glycerides were recovered from the washed ethereal solution and in all cases possessed a negligible iodine absorption (less than 0.2 %). Usually 500–600 g. of each fat was oxidised, 100 g. at a time, in order to provide an adequate quantity of purified fully-saturated glycerides for the detailed analysis of the component fatty acids present.

The crude fully-saturated glycerides thus obtained were further purified from any remaining acidic products of oxidation by adding a boiling saturated solution of potassium carbonate to the melted crude glycerides in an evaporating basin, with vigorous stirring, until a stiff paste was formed; this was immediately boiled with a large quantity of water until clarification was complete, allowed to settle, and the aqueous solution siphoned away from the upper layers of liquid fat and emulsion. This washing process was repeated until no emulsified paste was left, when a further washing with boiling aqueous potassium carbonate was given and the residual fat finally washed free from soap and alkaline carbonate by the above method. The alkaline and aqueous washings were collected together and thoroughly shaken with ether, and in this way two weighed fractions of fully-saturated glycerides were obtained, "A," the greater part of the whole, consisting of almost completely neutral fat, and "B," the product recovered from the ether extraction of the alkaline and aqueous washings, smaller in quantity than "A" and still somewhat contaminated with acidic material. The ether-extracted washings were treated with mineral acid and the acidic oxidation products were recovered: the acid value of the latter was observed and employed to estimate acidic products still present in fraction "B," on the assumption that the acid value of acidic

products in "B" was approximately the same as that of those removed during the purification.

These data suffice to determine the fully-saturated glyceride content of the original fat; from the saponification equivalents of the purified fully-saturated glycerides "A" and of the original butter-fat the molar percentages of fully-saturated glycerides follow, whilst, knowing the proportion of unsaturated acids present in the original fat from the earlier detailed analyses [1930, p. 1005], it is also possible to estimate the molar proportions of saturated acid present in the mixed saturated-unsaturated (and completely unsaturated) glycerides—the "association-ratio."

The results thus obtained for butter-fats I-V are collected in Table I.

Table I. *Determination of fully-saturated glyceride (F.S.G.) content.*

(i) <i>Analytical data.</i>										
Purification of crude fully-saturated glycerides										
No.	Weight taken g.	Crude F.S.G. g.	Weight taken g.	"A"		"B"		Acidic products		F.S.G. % (weight)
				g.	Acid No.	g.	Acid No.	g.	Acid No.	
I	601.9	182.4	61.8	42.2	0.5	9.5	8.8	9.9	153.9	25.4
II	606.5	248.0	244.2	204.6	0.3	26.7	6.5	12.9	170.0	38.5
III	501.2	183.3	181.3	150.8	0.4	24.6	9.9	5.9	149.5	35.05
IV	202.1	57.8	57.2	32.6	0.6	17.5	14.3	7.2	190.6	24.35
V	303.5	120.9	120.9	102.3	1.2	11.8	14.4	6.8	258.3	37.4

(ii) *Summary of observed values.*

No.	Iodine value	Acids in whole fat		Fully-saturated glycerides		"Association ratio" in non-fully-saturated part Mols. saturated acid per mol. unsaturated acid
		Saturated % (mol.)	Unsaturated % (mol.)	Weight %	Mols. %	
I	41.3	63.0	37.0	25.4	29.1	0.92
II	31.6	72.4	27.6	38.5	41.3	1.11
III	34.8	70.0	30.0	35.05	38.2	1.07
IV	41.6	61.9	38.1	24.35	27.2	0.94
V	34.5	70.2	29.8	37.4	39.6	1.07

Fatty acid compositions of fully-saturated glycerides of butter-fats I-V.

The purified fully-saturated glycerides "A" were converted into their mixed fatty acids and analysed as described in our former communication [1930], the operations being somewhat simplified by the absence of unsaturated acids and consequent omission of the lead salt separation of the non-volatile acids. An illustration of the results of this method of analysis in detail as applied to the whole acids of butter-fat IV was given in the paper referred to, and in the present cases only the final amounts of each acid as determined in the analyses are recorded.

Direct analysis of the saturated fatty acids combined in the acidic products of oxidation of mixed saturated-unsaturated glycerides cannot be effected owing to the presence of nonoic and azelaic acids as products of oxidation,

Table II. *Summarised analytical data for fatty acids from fully-saturated glycerides of butter-fats I-V.*

Acid	Volatile acids g.	Acids non-volatile in steam g.	Total g.	% (weight) % (mol.) (excluding unsaponifiable matter)	
Fully-saturated glycerides of butter-fat I.					
	8.39	92.7	101.09		
Butyric	4.70	—	4.70	4.7	11.7
Caproic	2.80	—	2.80	2.8	5.3
Caprylic	0.67	0.76	1.43	1.4	2.2
Capric	—	3.28	3.28	3.3	4.2
Lauric	—	4.79	4.79	4.7	5.2
Myristic	—	13.69	13.69	13.6	13.2
Palmitic	—	50.40	50.40	50.0	43.1
Stearic	—	19.60	19.60	19.5	15.1
Unsap.	0.22	0.18	0.40	—	—
Fully-saturated glycerides of butter-fat II.					
	15.45	175.2	190.65		
Butyric	7.21	—	7.21	3.8	9.2
Caproic	5.94	—	5.94	3.1	5.8
Caprylic	1.75	1.82	3.57	1.9	2.8
Capric	0.55	10.08	10.63	5.6	6.9
Lauric	—	19.73	19.73	10.4	11.1
Myristic	—	40.72	40.72	21.4	20.1
Palmitic	—	80.54	80.54	42.3	35.4
Stearic	—	21.87	21.87	11.5	8.7
Unsap.	—	0.44	0.44	—	—
Fully-saturated glycerides of butter-fat III.					
	10.72	114.6	125.32		
Butyric	4.67	—	4.67	3.7	9.2
Caproic	4.29	—	4.29	3.4	6.4
Caprylic	1.04	1.53	2.57	2.1	3.1
Capric	0.72	5.54	6.26	5.0	6.3
Lauric	—	7.40	7.40	5.9	6.4
Myristic	—	25.67	25.67	20.5	19.6
Palmitic	—	53.20	53.20	42.5	36.1
Stearic	—	20.63	20.63	16.5	12.6
Arachidic	—	0.50	0.50	0.4	0.3
Unsap.	—	0.13	0.13	—	—
Fully-saturated glycerides of butter-fat IV.					
	9.12	95.45	104.57		
Butyric	4.795	—	4.795	4.6	11.4
Caproic	2.815	—	2.815	2.7	5.1
Caprylic	0.88	0.94	1.82	1.7	2.7
Capric	0.63	3.71	4.34	4.2	5.3
Lauric	—	5.75	5.75	5.5	6.0
Myristic	—	16.41	16.41	15.7	15.1
Palmitic	—	48.17	48.17	46.2	39.5
Stearic	—	20.20	20.20	19.4	14.9
Unsap.	—	0.27	0.27	—	—
Fully-saturated glycerides of butter-fat V.					
	13.51	157.3	170.81		
Butyric	7.63	—	7.63	4.5	11.2
Caproic	4.09	—	4.09	2.4	4.6
Caprylic	1.51	2.22	3.73	2.2	3.4
Capric	—	6.84	6.84	4.0	5.1
Lauric	—	8.21	8.21	4.8	5.3
Myristic	—	26.27	26.27	15.4	14.9
Palmitic	—	79.11	79.11	46.5	39.9
Stearic	—	34.34	34.34	20.2	15.6
Unsap.	0.28	0.31	0.59	—	—

but the approximate composition of the unsaturated glycerides can be estimated by the difference between the respective acids present in the fully-saturated portion and in the whole fat as in Table III.

Table III. *General composition (weight) of butter-fats I-V.*

	Whole fat	Fully- saturated	Non-fully- saturated
Butter-fat I.			
	100 g.	25.4 g.	74.6 g.
Glyceryl residue $C_3H_5\equiv$	5.05	1.4	3.65
Butyric acid	2.95	1.1	1.85
Caproic "	1.65	0.65	1.0
Caprylic "	1.5	0.35	1.15
Capric "	2.0	0.8	1.2
Lauric "	3.25	1.15	2.1
Myristic "	6.55	3.25	3.3
Palmitic "	27.5	12.0	15.5
Stearic "	7.2	4.7	2.5
Arachidic "	0.85	---	0.85
Oleic "	38.0	---	38.0
Linoleic "	3.5	---	3.5
Butter-fat II.			
	100 g.	38.5 g.	61.5 g.
Glyceryl residue $C_3H_5\equiv$	5.2	2.15	3.05
Butyric acid	3.2	1.4	1.8
Caproic "	1.9	1.15	0.75
Caprylic "	1.05	0.7	0.35
Capric "	3.05	2.0	1.05
Lauric "	6.9	3.75	3.15
Myristic "	16.2	7.75	8.45
Palmitic "	25.6	15.4	10.2
Stearic "	4.55	4.2	0.35
Oleic "	30.05	---	30.05
Linoleic "	2.3	---	2.3
Butter-fat III.			
	100 g.	35.05 g.	64.95 g.
Glyceryl residue $C_3H_5\equiv$	5.2	1.95	3.25
Butyric acid	3.4	1.2	2.2
Caproic "	1.9	1.15	0.75
Caprylic "	1.6	0.7	0.9
Capric "	3.5	1.65	1.85
Lauric "	6.2	1.95	4.25
Myristic "	10.0	6.8	3.2
Palmitic "	24.8	14.1	10.7
Stearic "	7.9	5.45	2.45
Arachidic "	1.1	0.1	1.0
Oleic "	31.0	---	31.0
Linoleic "	3.4	---	3.4
Butter-fat IV.			
	100 g.	24.35 g.	75.65 g.
Glyceryl residue $C_3H_5\equiv$	5.05	1.35	3.7
Butyric acid	3.1	1.1	2.0
Caproic "	1.2	0.6	0.6
Caprylic "	1.15	0.4	0.75
Capric "	2.1	1.0	1.1
Lauric "	3.8	1.3	2.5
Myristic "	9.9	3.6	6.3
Palmitic "	24.8	10.6	14.2
Stearic "	6.2	4.4	1.8
Oleic "	38.8	---	38.8
Linoleic "	3.9	---	3.9

Table III (*contd.*).

	Whole fat	Fully- saturated	Non-fully- saturated
Butter-fat V.			
	100 g.	37.4 g.	62.6 g.
Glyceryl residue $C_3H_5\equiv$	5.15	2.05	3.1
Butyric acid	3.3	1.6	1.7
Caproic ..	1.6	0.85	0.75
Caprylic ..	1.25	0.8	0.45
Capric ..	2.9	1.4	1.5
Lauric ..	3.85	1.7	2.15
Myristic ..	10.55	5.45	5.1
Palmitic ..	25.9	16.45	9.45
Stearic ..	10.9	7.1	3.8
Arachidic ..	0.6	—	0.6
Oleic ..	29.75	—	29.75
Linoleic ..	4.25	—	4.25

DISCUSSION.

As already mentioned, we desire to review here the data for all the cow butter-fats which have now been submitted to examination by these methods, *i.e.* the present series of five specimens together with two former (market) samples of New Zealand butter and the recently-investigated specimen of Indian cow ghee. We thus have at disposal two Berkshire pasture-fed (late autumn and early summer), one Indian pasture-fed and three New Zealand (probably all pasture-fed) butters, with two other Berkshire butters from cows on winter diet supplemented by specific oil-cakes.

We find that our results lead to the consideration of two separate aspects of the glyceride structure of the butter-fats, namely, (i) the general distribution of unsaturated acids throughout the glycerides, which appears to be independent of the particular saturated acid components of the fat; and (ii) certain regularities in the occurrence of the saturated acid components in all the fats except those two in which added oil-cake formed a prominent part of the diet.

General distribution of unsaturated acids in the butter glycerides.

The data for all eight fats show consistently that the content of fully-saturated glycerides is, within close limits, simply a function of the relative proportions of saturated and unsaturated acids in the whole fat, irrespective of the nature of the saturated acids (the unsaturated acids are throughout almost constant in composition, *viz.* about 90 % oleic and about 10 % linoleic). In this respect the butter-fats resemble such other animal fats (beef and mutton tallows, lards, rabbit-fat) as have been examined, and also vegetable "pericarp" fats such as palm oil or stillingia tallow; all these are also not far removed in fully-saturated glyceride content (i) from glycerides of the same degree of unsaturation prepared synthetically by heating glycerol with a slight excess of the mixed fatty acids [*cf.* Bhattacharya and Hilditch, 1930], or

(ii) from that calculated on the assumption that the content of fully-saturated glycerides in mixed glycerides of saturated and unsaturated acids is proportional to the cube of the concentration of the total saturated acids. It will be remembered that vegetable seed-fats appear to be elaborated on entirely different and more "evenly distributed" lines [Collin and Hilditch, 1929].

The present series covers a relatively wide range of variation in the milk-fats from a single animal species and affords a more stringent comparison than has hitherto been possible of the relation of fully-saturated glyceride content to total saturated acid content in different specimens of a single type of fat. In Table IV will be found the iodine values and molar percentages¹ of saturated acids in the original butter-fats, together with the observed molar percentages of fully-saturated glycerides and (i) the latter calculated on the basis of proportionality to the cube of the total saturated acid present and (ii) the molar percentages of fully-saturated glycerides in synthetic mixed glycerides taken from the curve given by Bhattacharya and Hilditch [1930]; the "association ratio," or mols. of saturated acid present per mol. of unsaturated acid, in the non-fully-saturated glycerides is added in the final column.

Table IV. *Relationship between fully-saturated glyceride content and total saturated acid content of butter-fats.*

Butter-fat (cf. p. 507)	Iodine value	Fully-saturated glyceride content				"Association ratio" (observed)
		Total saturated acids % (mols.)	Observed % (mols.)	Calculated ($y \propto x^3$) % (mols.)	From synthetic glyceride curve (B and H) % (mols.)	
II	31.6	72.4	41.3	38.0	34.0	1.11
V	34.5	70.2	39.6	34.5	30.5	1.07
III	34.8	70.0	38.2	34.2	30.2	1.07
CG	36.0	67.9	33.7	31.5	27.5	1.07
A	38.0	67.3	33.8	30.5	26.5	1.04
B	39.4	66.1	31.5	28.7	25.0	1.03
I	41.3	63.0	29.1	24.5	21.5	0.92
IV	41.6	61.9	27.2	23.3	20.5	0.94

These relations are shown, probably more clearly, in Fig. 1, in which the appropriate portions of Bhattacharya and Hilditch's "synthetic" glyceride curve (unbroken line) and of the "calculated" curve (dotted) are plotted on an enlarged scale together with the observed values for the eight butter-fats. All the butter-fats contain rather more fully-saturated glycerides than would be the case if the fully-saturated glyceride content varied as the cube of the total saturated acids present, whilst, at this range of total unsaturation, the

¹ Throughout this discussion we are concerned with the relative number of molecules (or, perhaps, equivalents) of the various glycerides and fatty acids—the weight percentages usually employed are of little use in interpreting the structure of a complex mixture of glycerides of acids which differ widely (88 to 284) in molecular weight.

synthetic glycerides contain somewhat less fully-saturated components than the amount corresponding with this relationship. The relative regularity in the alteration of fully-saturated glyceride content with total unsaturation in the case of the butter-fats is perhaps more striking than the parallelism with either of the curves in question. The corresponding alteration in the proportions of saturated and unsaturated acids in the non-fully-saturated glycerides (Table IV, final column) should also be noted¹.

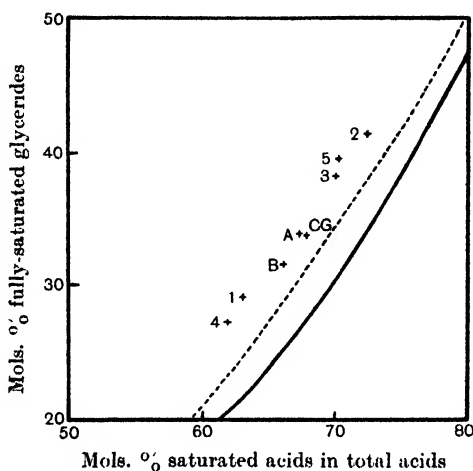


Fig. 1.

These data are considered to show that, as between saturated and unsaturated acids, the constitution of the mixed glycerides in butter-fat is determined by the relative concentration of these classes of acid; this proportion being given in any particular case, the mode of assemblage into triglyceride molecules appears to follow according to some fixed rule. Although the experimental methods only permit of differentiation between saturated and unsaturated acids, there is no reason to suppose that, normally, the case is different as between one saturated acid and another.

As regards the general composition of the non-fully-saturated glycerides of the eight fats, the molar "association ratios" in the final column of

¹ By way of contrast, the corresponding available data for the two seed-fats so far examined [Collin and Hilditch, 1929] in this region of relative unsaturation may be recalled:

Fat	Iodine value	Total saturated acids % (mols.)	F.S.G. content % (mols.)	"Association ratio"
Cacao butter	37.1	59.8	2.5	1.4
Illipé tallow	32.3	62.8	4.5	1.55

In seed-fats, so long as there is sufficient saturated acid present, there is a tendency towards a constant association of about 1.4 mols. saturated with 1 mol. of unsaturated acid in the "mixed" glycerides and, at this particular state of relative unsaturation, the content of fully-saturated glycerides is exceedingly small.

Table IV permit limiting values to be calculated for the possible proportions of either mono-unsaturated di-saturated and di-unsaturated mono-saturated glycerides, or mono-unsaturated di-saturated and tri-unsaturated glycerides which may be present; in the absence of any analytical method which will allow the amount of any one of these classes (*e.g.* triolein) to be determined, this is as far as we can go at present. On the other hand, there is little or no evidence that triolein occurs in any great amount in butter-fat, and considerable indirect evidence against this possibility [*cf.* Amberger, 1918]; and very probably the minimum limits for mono-unsaturated derivatives and the maxima for the di-unsaturated class given in Table V are not far removed from the actual proportions present.

Table V. *Limiting values for the molar percentages of the four types of mixed glycerides in butter-fats.*

Butter	Iodine value	"Association ratio"	Fully saturated	Glycerides (mols. %)		
				Mono-unsaturated-di-saturated	Di-unsaturated-mono-saturated	Tri-unsaturated
II	31.6	1.11	41.3	33.0-46.3	24.8-0	0-12.4
V	34.5	1.07	39.6	33.3-46.8	27.1-0	0-13.6
III	34.8	1.07	38.2	34.0-47.9	27.8-0	0-13.9
CG	36.0	1.07	33.7	36.5-51.4	29.8-0	0-14.9
A	38.0	1.04	33.8	35.0-50.6	31.2-0	0-15.6
B	39.4	1.03	31.5	35.8-52.1	32.7-0	0-16.4
I	41.3	0.92	29.1	31.0-51.0	39.9-0	0-19.9
IV	41.6	0.94	27.2	33.0-52.9	39.8-0	0-19.9

Whilst these general characteristics of butter-fat glyceride structure may prove to be of some importance with regard to the biochemical elaboration of milk-fats, they also have a practical bearing on the variation in the "melting point" or softness of butters. It is evident that increase in oleic acid content of butter, according to the above data, will be reflected in a disproportionate increase in softness, since not only is the ratio of unsaturated to saturated acids increased in the saturated-unsaturated mixed glycerides, but the quantity of the latter increases considerably with augmentation of the oleic acid. For example, in 100 mols. of fat, butter-fat IV contains about 73 mols. of mixed glycerides in which the unsaturated acids are in excess of the saturated, whereas butter-fat II contains only about 59 mols. of mixed glycerides in which there are 11 mols. of saturated acid for every 10 of unsaturated. Whilst, therefore, the fully-saturated glycerides (which are of course the highest-melting components of butter) have been reduced by 14 mols. in every 100 of total fat, the mixed glycerides contain relatively more combined oleic acid, again leading to the production of components of lower melting point. These data also illustrate the obvious reasons for the varying differences which obtain between the melting or softening point of most natural fats and the corresponding (and usually much higher and sharper) melting points of their respective mixed fatty acids; the melting points of individual simple triglycerides being, on the other hand, of the same order

and usually somewhat higher than those of the fatty acid from which they are derived [Joglekar and Watson, 1930].

Distribution of the individual fatty acids in the butter-glycerides.

In order to discuss several features of this aspect of the glyceride structure it will be necessary to refer to the molar percentages of each acid combined in the whole fat, the fully-saturated glycerides and the remaining glycerides; these are collected in Tables VI, VII and VIII. The butter-fats are arranged in increasing order of total unsaturation except that, since the fats II and III (from cows whose food contained large amounts of specific oil-cakes) show certain differences from the remaining (pasture-fed) fats, the data for samples II and III are placed at the end of each table.

Table VI. *Molar distribution of fatty acids in the whole fat.*

Fat	...	V	CG	A	B	I	IV	II	III
Butyric acid		9.2	6.9	9.2	8.4	8.4	8.9	9.0	9.6
Caproic	"	3.4	4.0	3.7	3.9	3.5	2.7	3.9	3.0
Caprylic	"	2.2	2.2	1.4	1.3	2.7	2.0	1.7	2.8
Capric	"	4.2	4.9	2.7	2.8	2.9	3.0	4.3	5.1
Lauric	"	4.7	6.7	3.7	4.6	4.1	4.7	8.3	7.5
Myristic	"	11.5	10.9	10.2	11.0	7.2	10.9	17.2	10.7
Palmitic	"	25.0	26.8	25.7	26.2	27.1	24.3	24.1	23.7
Stearic	"	9.5	5.5	10.2	7.1	6.4	5.4	3.9	6.7
Arachidic	"	0.5	—	0.5	0.8	0.7	—	—	0.9
Oleic	"	26.1	28.4	28.9	30.8	33.9	34.6	25.7	27.0
Linoleic	"	3.7	3.7	3.8	3.1	3.1	3.5	1.9	3.0

Table VII. *Molar distribution of fatty acids in the fully-saturated glycerides.*

Fat	...	V	CG	A	B	I	IV	II	III
Molar % F.S.G.	...	39.6	33.7	33.8	31.5	29.1	27.2	41.3	38.2
Butyric acid		11.2	11.2	11.0	10.5	11.7	11.4	9.2	9.2
Caproic	"	4.6	5.1	6.5	4.9	5.3	5.1	5.8	6.4
Caprylic	"	3.4	0.5	1.8	5.0	2.2	2.7	2.8	3.1
Capric	"	5.1	4.4	3.3	3.1	4.2	5.3	6.9	6.3
Lauric	"	5.3	6.1	4.1	4.7	5.2	6.0	11.1	6.4
Myristic	"	14.9	15.5	17.9	17.0	13.2	15.1	20.1	19.6
Palmitic	"	39.9	43.0	39.6	39.3	43.1	39.5	35.4	36.1
Stearic	"	15.6	14.2	15.8	15.2	15.1	14.9	8.7	12.6
Arachidic	"	—	—	—	0.3	—	—	—	0.3

Table VIII. *Molar distribution of fatty acids in the mixed saturated-unsaturated glycerides.*

Fat	...	V	CG	A	B	I	IV	II	III
Molar % M.S-U	...	60.4	66.3	66.2	68.5	70.9	72.8	58.7	61.8
Butyric acid		7.9	4.8	8.3	7.3	7.2	7.8	8.6	9.6
Caproic	"	2.6	3.4	2.3	3.3	2.9	2.2	2.7	2.5
Caprylic	"	1.3	3.1	1.1	—	2.8	1.8	1.0	2.4
Capric	"	3.6	5.1	2.3	2.6	2.4	2.2	2.4	4.1
Lauric	"	4.4	7.0	3.4	4.7	3.7	4.3	6.4	8.2
Myristic	"	9.2	8.6	6.4	8.4	5.0	9.4	15.1	5.4
Palmitic	"	15.0	18.6	18.9	20.1	20.9	18.7	16.3	16.1
Stearic	"	5.6	1.1	7.5	3.3	3.1	2.2	0.5	3.3
Arachidic	"	1.0	—	0.7	1.1	1.0	—	—	1.2
Oleic	"	43.2	42.7	43.4	44.8	46.7	46.7	43.7	42.5
Linoleic	"	6.2	5.6	5.7	4.4	4.3	4.7	3.3	4.7

Further, we have found it convenient to compare the component acids in the following groups: (i) the lower acids from butyric to lauric inclusive, (ii) myristic and palmitic; and (iii) the total amount of acids containing 18 carbon atoms in the molecule (the very small quantities of C_{20} acids recorded in some of the analyses are included with this group). The molar percentages of these groups present in each fat and in its two subdivisions are shown in Table IX.

Table IX. *Molar distribution of butyric-lauric, myristic-palmitic, and C_{18} acids in the butter-fats.*

	Fat	V	CG	A	B	I	IV	II	III
Section	Acids										
Whole fat	{ Butyric-lauric			23.7	24.7	20.7	21.0	21.6	21.3	27.2	28.0
	{ Myristic-palmitic			36.5	37.7	35.9	37.2	34.3	35.2	41.3	34.4
	{ C_{18} acids			39.8	37.6	43.4	41.8	44.1	43.5	31.5	37.6
Fully saturated	{ Butyric-lauric			29.6	27.3	26.7	28.2	28.6	30.5	35.8	31.4
	{ Myristic-palmitic			54.8	58.5	57.5	56.3	56.3	54.6	55.5	55.7
	{ Stearic			15.6	14.2	15.8	15.5	15.1	14.9	8.7	12.9
Saturated-unsaturated	{ Butyric-lauric			19.8	23.4	17.4	17.9	19.0	18.3	21.1	26.8
	{ Myristic-palmitic			24.2	27.2	25.3	28.5	25.9	28.1	31.4	21.5
	{ C_{18} acids			56.0	49.4	57.3	53.6	55.1	53.6	47.5	51.7
	{ (Oleic-linoleic			49.4	48.3	49.1	49.2	51.0	51.4	47.0	47.2)

The six butter-fats from cattle on normal diets. The cows from various districts which were fed on pasture (or on pasture with roots, etc., but no added oil-cake) yielded butters which show much similarity in many features of their glyceride structure.

(i) In the total fatty acids of each whole butter-fat (Table VI), the amounts of (a) butyric-lauric, (b) myristic-palmitic and (c) stearic, oleic and linoleic acids tend severally towards more or less constant values. This approximate constancy extends to some of the individual acids, especially palmitic, myristic and perhaps butyric; but the amount of stearic acid is more variable. Yet the total amount of C_{18} acids is of much the same order throughout, variations due to the substantially different amounts of unsaturated acids in the butters being counterbalanced partly by variation in the amount of stearic acid, partly by the gross effect of minor variations in all the other saturated acids.

(ii) Although, as the total unsaturation in the fats varies, the amount of fully-saturated glycerides differs widely throughout the series, their composition (Table VII) is remarkably constant; especially noteworthy is the regular proportion of stearic acid (15 ± 0.8 mols. %) in contrast to its variability (5–10 mols. %) in the whole fats. All the saturated acids are present in both fully- and non-fully-saturated parts of the fats, but the tendency noticed by Hilditch and Jones [1929] for the lower acids to associate slightly more than the higher saturated acids with the unsaturated acids is confirmed by the present more extended results.

The inference can fairly be drawn, for these "normal diet" butters, that,

whilst the whole fats vary in unsaturation over a range of 34.5–41.3 in iodine value (61.9–70.2 mols. % total saturated acids), the composition of the fully-saturated glycerides (which comprise from 27 to 40 mols. % of the whole fat) is in every case much the same.

(iii) In the non-fully-saturated glycerides (Table VIII), some approximation to constancy in the molar content of the three groups of acids mentioned follows automatically from the general tendencies noted in (i) and (ii) above. The proportion of unsaturated acids (Table IX, last line) increases slowly, but on the whole steadily, with the total unsaturation of the butter-fats as a whole, but in less than direct proportion to the molar percentage of unsaturated acids present in the whole fat.

(iv) The degree of concordance shown in the totals of the molar contents of butyric-lauric acids is of special interest in view of the importance of these acids as the characteristic acids of milk-fats. In the whole fats this figure appears to tend to a constant value of about 21–22, four of the six fats giving values between 20.7 and 21.6 (Table VI). The maximum variation in the combined butyric-lauric acid molar content of the six butter-fats is, indeed, much less than that in the corresponding Reichert-Meissl values; it is not unlikely that, with a reasonably rapid analytical method for the determination of combined butyric-lauric acid content, this figure would be a more certain index for milk-fats than the Reichert-Meissl value. We are endeavouring to ascertain whether a simplified procedure can be devised by means of which a measure of the total butyric-lauric acid content may be more readily obtained than by employment of the full "fractionation" analysis.

We might not have felt justified, at this stage, in stressing some of the foregoing likenesses in glyceride structure of these butters except for the fact that an examination of a number of beef tallowes from different sources, now being made by Mr A. Banks in this laboratory, is yielding results of a similar and equally concordant nature. In the tallow investigation, an account of which it is hoped to publish at an early date, the number of individual component acids to be considered is much less than in butters and the regular features which we have been discussing stand out more sharply in consequence. At the same time, notwithstanding the complexity of the fatty acid mixture in butters, inspection of the foregoing tables on the lines suggested by this more recent work on the body-fats has shown that similar influences operate in the case of the milk-fats from pasture-fed sources.

It is interesting to find, further, that the combined molar contents of myristic and palmitic acids in the total butter-fats, and in their fully-saturated portions, are only a few mols. % lower than the corresponding average values for beef tallowes. In other words, the molar contents of C_{18} acids in the tallowes (a) as a whole or (b) in their fully-saturated components are respectively within a few units of the molar contents of all the acids, other than myristic and palmitic, (a) in butter-fat or (b) in its fully-saturated components. This naturally suggests that the lower fatty acids characteristic of butter are

elaborated at the expense of acids of the stearic series. We are not disposed to assert that the lower fatty acids present in butter necessarily result directly from stearic or oleic acid, but the data which we have put forward are definitely consistent with the view that a precursor of stearic or oleic acid in a tallow may, in the course of milk-fat metabolism, appear instead as a lower fatty acid, with the proviso that only one molecule of any of the lower acids is produced in milk-fat for each molecule of stearic (oleic) acid which otherwise would have resulted in a body-fat. We also consider that the present data confirm our former view that palmitic and (possibly) myristic acids stand apart from the remaining butter acids in many respects, and in particular we find it difficult at present to suppose that the sources of palmitic acid and of oleic, stearic, *etc.*, acids in the natural fats can be identical.

At first glance it may seem difficult to reconcile these regularities in glyceride structure with the variations in the component fatty acids of the same series of butter-fats which we discussed in our previous paper and which we showed to be somewhat large, and due more to conditions such as the change from outdoor to indoor life, general type of diet, or seasonal changes of temperature than to added fats given in the diet. It was pointed out, however [1930, p. 1110], that the chief variations due to such causes were in the content of unsaturated acids and of stearic acid, *i.e.* on the whole, in the balance between stearic, oleic and linoleic acids. Since the amount of fully-saturated glycerides depends on the relative amount of unsaturated acids present in the whole fat, this is a variable, indeed almost the only markedly variable, feature in the glyceride structure of the butters; for the rest, the assemblage of fatty acids into glycerides evidently operates in the same way whatever may be the individual acids concerned (the manner being very similar for such animal fats and vegetable "pericarp" fats as have been studied, but different in the case of vegetable seed-fats). Thus, when the factor due to variable unsaturation (the varying fully-saturated glyceride content) has been taken into account, close similarities are revealed in the respective fully-saturated and non-fully-saturated components of the butters—the constancy of the stearic acid content of the fully-saturated portions being, perhaps, the most illuminating feature.

The two butter-fats from cows whose diet included specific oil-cakes.

By way of contrast, the differences due to inclusion in the diet of coconut or soya bean cake appear more clearly from the component glyceride structures than from simple consideration of the total fatty acids of butters II and III [1930, p. 1111]; this will be seen by comparing the data in Tables VI–IX for Nos. II and III with those for the other six butters.

(i) *Coconut cake in diet (butter II)*. It was pointed out previously [1930] that the only distinctive features in the total acids of butter-fat II were increased amounts of myristic and lauric acids, with, however, less augmentation in the lauric, capric and caprylic acids than might have been anticipated

in view of the high lauric acid and appreciable capric and caprylic acid contents of coconut oil.

In the fully-saturated components of butter-fat II, however, the relative differences are more sharply defined: the lauric acid molar content (Table VII) is 11 (instead of about 5-6) and the myristic acid molar content is 20 (against 15-17). Since dilauromyristin is probably the major component of coconut oil [Collin and Hilditch, 1929], the figures suggest that to some extent this glyceride has passed through directly into the milk-fat, an inference which is supported by consideration of the butyric-lauric acid contents of the fully-saturated and non-fully-saturated parts of the fat.

The butyric-lauric acid content (Table IX) as a whole is much higher (35.8 mols. %) than in the fully-saturated components of the six "normal" butters (27-30 mols. %), whilst the palmitic and stearic acid contents are correspondingly low; the latter (9 mols. %) is in sharp contrast to the steady value of about 15 mols. % observed in the pasture-fed butters (fully-saturated glycerides). Thus the amounts of the individual components of the fully-saturated part of butter-fat II (except capric and caprylic acids) differ distinctly from those in the six butter-fats discussed above. On the other hand, the combined butyric-lauric acid content (21.1 mols. %) of the non-fully-saturated glycerides of butter-fat II is only slightly higher than the corresponding values for the normal fats—indeed the only notable divergence from the normal is an increase in the myristic acid and a minor increase in lauric acid. Consequently, practically all the differences in the butter-fat, caused by feeding coconut-fat (which contains about 85 % of fully-saturated components) to the cows, have been traced to variations from the normal composition in the fully-saturated part of the butter. Whilst, therefore, these more intimate analyses support our earlier conclusion that the greater part of the characteristic components of the coconut-fat have not appeared in the milk-fat, they clearly indicate the location of certain portions of the typical coconut fully-saturated glycerides which have, apparently, passed unchanged into the milk.

(ii) *Soya bean cake in diet (butter III)*. It has already been emphasised [1930] that the relatively large amount of linoleic acid in soya bean oil has little appreciable effect on the content of linoleic and oleic acids in the butter-fat, which is of the normal order. Examination of the component glycerides reveals (Table IX) that in butter III the butyric-lauric acid content of the non-fully-saturated components (26.8 mols. %) is much higher than the average value for the six "normal" butters (18-20 mols. %); myristic and stearic acids (Table VIII) are about normal and palmitic acid (16.1 mols. %) is definitely below the normal (18-20 mols. %). On the other hand, the glyceride structure of the fully-saturated part (except for a somewhat large content of myristic acid) is not far from the normal range of the six pasture-fed butters.

Thus whilst ingestion of the relatively highly saturated coconut-fat led

to variation from the normal in the character of the fully-saturated components of butter-fat II, the diet including soya bean oil (nearly all of which consists of unsaturated glycerides) has caused differences from the normal which are for the most part confined to the non-fully-saturated glycerides of butter-fat III. In the latter case, however, the observed differences do not involve any increase in the proportion of linoleic acid (the major component of soya bean oil) but are confined for the most part to augmentation of the butyric-lauric acid group; thus suggesting that the latter may, in part, be metabolic products from the soya bean oil acids which remain combined in the mixed saturated-unsaturated glycerides.

It will be recalled that the New Zealand December pasture-fed butter-fat V, which has almost the same iodine value as butter-fat III from the soya bean cake diet, resembles the latter in much of its total fatty acid composition [Hilditch and Sleightholme, 1930, p. 1110]; but the above study of the glyceride structure reveals the differences which in reality exist between the two fats. Indeed, unless investigation of glyceride structure be undertaken it appears that sufficient data cannot be provided for the adequate consideration of milk-fat metabolism.

SUMMARY.

Four English butter-fats from members of the same herd of cows fed on various diets and a New Zealand pasture-fed (December) butter-fat (the total fatty acid compositions of which have been previously discussed) have been oxidised in order quantitatively to determine their content of fully-saturated glycerides, and the fatty acids of the latter have been analysed in detail. The results obtained, with similar data for two other New Zealand butter-fats and an Indian cow ghee, are discussed with reference to the general glyceride structure of butter-fats.

The six butters from cows on "normal" (usually pasture-fed) diets show many features in common, whilst in two cases in which specific fatty oils had been fed to the cows the results stand apart in certain respects; but all eight fats conform to the usual rule (in animal fats) that the content of fully-saturated glycerides is a function of the proportion of the total saturated acids present in the mixed acids of the whole fat.

In the "normal" butters, moreover, the component acids of the fully-saturated parts are present in approximately the same quantitative amounts, the composition of the fully-saturated glycerides tending to be the same whatever the original unsaturation of the butter as a whole. Similar concordances are revealed in the combined butyric-lauric acid contents, and in the combined myristic-palmitic acid contents, of the whole fats and of their two divisions, fully- and non-fully-saturated glycerides, whilst the content of unsaturated acids in the non-fully-saturated glycerides increases steadily and slowly with increase of unsaturation in the fats as a whole.

When coconut-fat (a relatively saturated fat) formed part of the diet, the

fully-saturated part of the butter showed a marked increase in butyric-lauric acid content, but for the most part the composition of the non-fully-saturated glycerides was normal. With a soya bean (predominantly unsaturated) oil diet the structure of the butter glycerides was different: the fully-saturated components were not far removed from the normal in composition, but the lower acids of the non-fully-saturated portion were present in excess of the usual proportion.

Study of the glyceride structure of these eight butters, whilst confirming the general rules which have been observed to connect the amount of fully-saturated glycerides with the degree of unsaturation of animal fats, thus reveals influences due to diet more clearly than the consideration of the component fatty acids of the whole fats, and affords some additional evidence as to the mode of utilisation of ingested fat in milk-fat metabolism.

We have again to express our grateful appreciation for the assistance given to us by Captain Golding and the National Institute for Research in Dairying and to thank Messrs Lever Brothers, Ltd., for a Research Studentship held by one of us (J.J.S.) during the course of the work.

REFERENCES.

- Amberger (1918). *Z. Unters Nahr. Genussm.* **35**, 313.
Bhattacharya and Hilditch (1930). *Proc. Roy. Soc. Lond. A* **129**, 468.
—— (1931). *Analyst*, **56**, 161.
Collin and Hilditch (1929). *Biochem. J.* **23**, 1273.
Hilditch and Jones (1929). *Analyst*, **54**, 75.
—— and Sleightholme (1930). *Biochem. J.* **24**, 1098.
Joglekar and Watson (1930). *J. Ind. Inst. Sci.* **13 A**, 119.

LXII. THE EFFECT OF HYDROGEN ION CONCENTRATION ON SOME OXIDATIONS BY *B. COLI COMMUNIS*.

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THE effect of treatment with toluene on the aerobic and anaerobic oxidations of various substrates has been noted in a previous communication [Cook, 1930]. The present paper is the outcome of experiments which were undertaken to investigate the most favourable conditions of hydrogen ion concentration for the aerobic and anaerobic oxidations of formate, succinate and lactate. Apart from the immediate practical value of these experiments, the results are of interest in relation to the kinetics of the action of oxidative enzymes and the interaction of the dehydrogenases and oxygenases in the bacterial cell.

The p_{H} -activity curves of the oxidases have not been studied in such great detail as those of the hydrolytic enzymes. For a summary of work on the latter Haldane's monograph [1930] should be consulted. Ohlsson [1921] made a very careful study of extracted muscle succinoxidase, the results of which in relation to the present work will be discussed later. Quastel and Whetham [1924] investigated the effect of p_{H} on the dehydrogenation of succinate by normal *B. coli*. Dixon and Thurlow [1924] and Bernheim [1928] studied the p_{H} -activities of xanthine oxidase and the potato aldehyde oxidase respectively. In all the above instances, with the exception of one experiment by Bernheim who used nitrate, methylene blue was used as the hydrogen acceptor. There appears to be little work on the oxidases in which oxygen either activated or not is involved. Hare [1928] made an interesting study of the p_{H} -activity curve of tyramine oxidase in which unactivated oxygen is involved. Cook and Stephenson [1928] studied the effect of p_{H} on the oxidation of lactate by activated oxygen by normal *B. coli*. References to peroxidase, catalase and some other oxidases are given in Haldane's monograph.

It had been noted earlier [Cook, 1930] that above a temperature of about 30° the rate of oxygen reduction appeared to be the limiting factor in the aerobic oxidation of formate and lactate. The aerobic oxidation of these substrates was therefore studied at 16° and 40°. The anaerobic experiments in which the reduction of methylene blue in the Thunberg tube was observed and which are a measure solely of the dehydrogenases were carried out at 40°.

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From the earlier results it was shown that the reaction velocity increased in a linear manner with increase of temperature until destruction of the enzyme occurred at a temperature of about 50°. Some experiments carried out at a lower temperature showed that the shape of the p_{H} -activity curves remained substantially the same as that of those at 40°.

EXPERIMENTAL.

The bacterial suspension used for these experiments was derived from a strain of *B. coli communis* Escherich obtained from the National Collection of Type Cultures. Some experiments were carried out on a strain obtained from the Pathology Department, Cambridge, but apart from slight differences in the velocities of respiration no significant differences were noted in the shapes of the curves. The method by which the stock preparation is prepared has been described previously [Cook, 1930]. This preparation retains its activity for several weeks if kept in the refrigerator, but if left in the open autolysis sets in and it rapidly deteriorates. The effect is most marked on the reduction of oxygen. The reduction of methylene blue especially by formate and lactate shows only a slight falling off in activity. From the stock preparation of bacteria the toluene-treated organism is prepared by making a suitable dilution so that a convenient velocity may be obtained. The dilution is best made with frog Ringer's solution containing 0.65 % NaCl, 0.014 % KCl and 0.012 % CaCl_2 . No NaHCO_3 is added as it would cause an increase in p_{H} due to loss of CO_2 . It has been found that, while preparations in which the dilution has been made with water are apt to show some falling off in activity after several hours, those made up with Ringer's solution keep well even at room temperature for 6–8 hours, which is well within the time necessary for one series of experiments to be made. The preparation is kept on ice during the course of the experiments. With regard to the action of the Ringer's solution it is necessary to note that the effect of the added salts on the shape of the curves is very slight indeed. The actual amount of salts present in the aerobic experiments is a third of those present in normal Ringer's solution and in the anaerobic experiments at most a tenth. If a comparison is made of the p_{H} -activity curve of the methylene blue reduction in the presence of well-washed bacteria in distilled water and bacteria in the presence of a concentration of salts corresponding to Ringer's solution, the velocity is reduced by about 10 % in the latter case, but the curve follows that obtained for the washed preparation. The aerobic oxidations show a very slight difference between the initial rates of respiration, but a difference is noted in the activity after several hours. The activity of the washed preparation has fallen by about 15–20 % after 6 hours. This difference is not nearly so marked with the reduction of methylene blue, so that some alteration in the oxygenase is possibly indicated. The study of the oxidation of succinate is complicated by the fact that after two or three days the organism loses its capacity for the aerobic oxidation at 16°. Fresh preparations of bacteria oxidise succinate at

this temperature and the oxidation at 40° and the methylene blue reduction are unaffected. The technique followed for the aerobic and anaerobic experiments is essentially the same as that described previously.

The buffers used were the Clark-Lubs series of potassium hydrogen phthalate-NaOH, KH_2PO_4 -KOH and boric acid-KCl, NaOH. A borax- KH_2PO_4 buffer (Kolthoff) was also employed. This buffer is to be highly recommended as it may be used to check any effects of the phosphate or borate ions *per se* over a considerable portion of their range. The p_H of the reaction mixtures was checked electrometrically at the beginning and end of numerous experiments. The p_H of the undiluted bacterial suspension was in general about 7.4. The bacteria if present in large amounts have a considerable buffering capacity but, as they are used in relatively small amounts in the actual experiments and the solutions of buffer added were always present in a concentration of at least $M/120$, the p_H of the mixture remained practically that of the buffer solutions. The substrate concentration and the total nitrogen of the bacteria present are given in the separate experiments. The substrate concentration taken was always very much greater than that necessary for saturation of the enzyme. The total nitrogen of the bacteria may be used as a measure of the dry weight of bacteria present, as noted previously.

As a test of the aerobic method the following experiment was undertaken. A preparation of bacteria was made by dilution of the stock with Ringer's solution and treated with toluene for exactly half an hour. This time was made the standard time for treatment. The procedure followed was to shake the suspension well with toluene at the beginning of the treatment and to shake at intervals of 5 minutes. The treatment was always made at room temperature. The lower layer of bacteria was then pipetted free from the toluene layer. A small amount of toluene is always present in the material used for experiment and although this does not seem to affect the anaerobic experiments there is a possibility that the presence of the toluene would affect the manometric readings. The same amount of bacterial suspension was therefore placed in both cups of the Barcroft compensating manometer. The suspension thus prepared was diluted to various extents, and the oxidation of lactate at p_H 7.6 and 40° was studied. After 4 hours the stock suspension of the treated organism was again diluted and the experiments repeated. The results are shown on Fig. 1. It will be seen that the velocity of the oxidation is almost proportional to the amount of bacteria. It will also be noted that there is no significant difference between the results obtained half an hour and 4 hours after treatment with toluene.

Anaerobic experiments.

The reduction of methylene blue at 40° in the Thunberg tube in the presence of a standard concentration of substrate and a known amount of bacteria was taken to a standard of 90 % reduction of the methylene blue present. The concentration of this dye used throughout was 1/30,000 for each experiment.

The toluene-treated organism does not reduce methylene blue in the absence of substrate even after several days. A standard consisting of the same amount of bacteria used in the experiments and an amount of dye corresponding to 90 % reduction may be preserved for some time. Fig. 2 represents the oxidation of formate by methylene blue at 40°. The buffer solutions used for obtaining various parts of the curve are indicated on the figure. It will be seen that the curve from p_H 4 to 7 rises steadily. There is an optimum at about

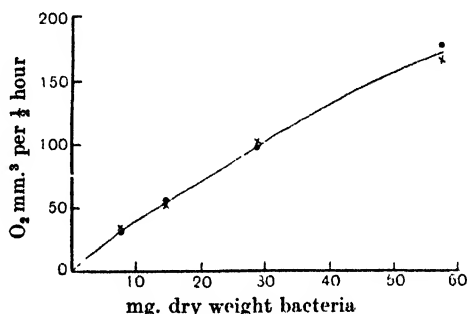
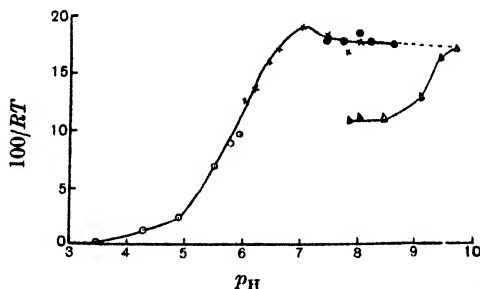


Fig. 1. The aerobic oxidation of *M/60* lactate at p_H 7.6 and 40°.

Ordinate: oxygen uptake in initial 30 minutes.

Abscissa: dry weight of bacteria in mg.; obtained by taking the total nitrogen as representing 8.3 % of the dry weight of the bacteria.

Values shown by dots represent the velocities half an hour after treatment and those shown by crosses 4 hours after treatment.



○-○-○ Phthalate. ×-×-× Phosphate. ⊗-⊗-⊗ Borax-Phosphate. △-△-△ Borate.

Fig. 2. The anaerobic oxidation of *M/120* formate by toluene-treated *B. coli* in the presence of 1/30,000 methylene blue at 40°.

Ordinate: velocity, this being the reciprocal of the reduction time for 90 % reduction of the methylene blue $\times 100$.

Abscissa: p_H .

Bacterial nitrogen present for each experiment 0.192 mg.

p_H 7 after which the curve falls slightly, rising again though not to the same level from p_H 7.8 to 9.0. It will also be noted that the velocity in borate buffer at p_H 7.8 is considerably less than in phosphate at this point. In the more alkaline region the curve falls rapidly owing to irreversible inactivation of the enzyme as will be shown later. Fig. 3 shows the anaerobic oxidation of lactate, and it will be seen that the curve is similar in shape to the formate curve, but no peak is to be noted. Fig. 4 represents the anaerobic oxidation of succinate. The curve is of the same shape as that obtained for lactate but

the maximum is not reached until a p_H of 8.6, and the plateau is only of short duration.

The effect of the borate buffer at the same p_H as the phosphate buffer is to be noted, though in a different degree, with all three dehydrogenases. It was at first thought that the difference in velocity at the point where the p_H was the same might be explained by (a) an inhibiting effect of the borate ion or (b) an accelerating effect of the phosphate ion. But this latter effect is

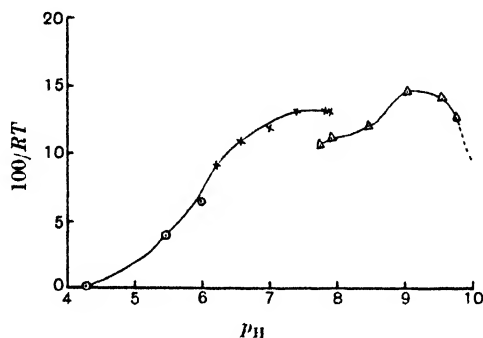


Fig. 3. The oxidation of *M*/120 lactate at 40°. Ordinate and abscissa as for Fig. 2. Bacterial nitrogen 0.48 mg.

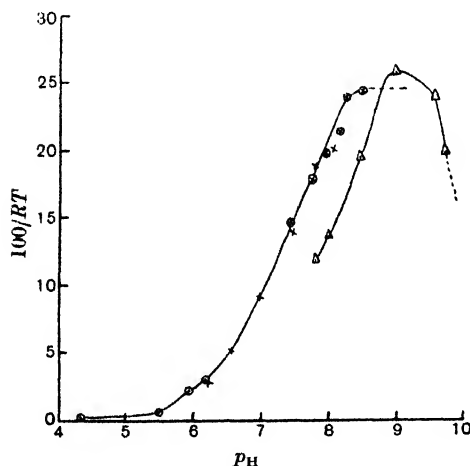


Fig. 4. The oxidation of *M*/120 succinate at 40°. Bacterial nitrogen 0.96 mg.

rendered unlikely in that no appreciable difference is noted at the change from phosphate to phthalate buffers. Experiments carried out with a borax-phosphate buffer gave a velocity equal to that obtained for the phosphate buffer. The effect of the borate buffer is not due to the presence of the KCl. Experiments carried out with diluted borate buffer and phosphate buffer to which KCl was added did not show any alteration in velocity. It will be noted that the curve obtained with borate rises to give approximately the same

velocity as that for borax-phosphate buffer. The effect of the borate buffer is always most marked at p_H 7.8-8.0. It would perhaps seem that the decrease in velocity was due to an effect of the undissociated boric acid. The p_K of boric acid is 9.24 so that at p_H 7.8 the acid is only slightly dissociated.

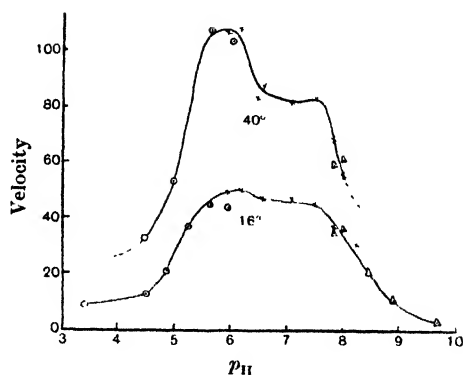
Aerobic oxidations.

The toluene-treated organism has no endogenous oxidation, so that the results obtained represent oxidation of the substrates only. The method employed has been described previously. The Barcroft manometers when set up were swung open to the air for exactly 5 minutes. The taps were then shut and the initial readings taken. Readings were then taken at 15-minute intervals. The velocity was obtained from the difference in the readings after 15 and 30 minutes. A reading at 45 minutes served to show whether the velocity was still constant. At the more acid and alkaline p_H values the velocity fell off indicating enzyme inactivation. At values ranging from p_H 5 to 9 there was no appreciable diminution. The p_H was checked electrometrically at the beginning and end of the experiments. There is no appreciable alteration in the oxidation of lactate and succinate, but with formate there is a change of about 0.2 of a unit with the concentration of buffer used. This change is due to the fact that the formate is oxidised to bicarbonate. This dissociates at p_H 7.4, the CO_2 being taken up by the KOH in the manometer. Thus the effect on p_H of the oxidation of formate is the same as that of adding an equivalent of NaOH. The actual change in p_H during the course of the experiment is probably not more than 0.1 of a unit. The extra difference was probably due to the fact that further oxidation had occurred during the interval between removing the solution from the manometer pot and measuring the p_H . At the time when the velocity was measured, oxidation of 50 % at most of the formate had taken place. This would raise the p_H of $M/20$ buffer solution by about 0.1 of a unit. In general this figure has been added to the initial p_H reading of the aerobic formate experiments.

Figs. 5, 6 and 7 represent the aerobic oxidations of formate, lactate and succinate respectively. It will be seen that the curves obtained for formate and lactate at 16° and 40° show little difference in shape with increase of temperature. It will also be noted that there is practically no difference between the velocities of oxidation where the change is made from phosphate to borate buffer. A similar result was found by Cook and Stephenson [1928] for the aerobic oxidation of lactate by the untreated organism.

Reversibility of the effect of acid and alkaline p_H on the aerobic and anaerobic systems.

It is necessary before any attempt can be made to interpret the nature of the curves to observe the action of the more acid and alkaline buffers on the reactions. The curves show that the velocity is considerably diminished at p_H 3 and 10. The procedure adopted was as follows: 50 cc. of the toluene-



○-○-○ Phthalate. ×-×-× Phosphate. △-△-△ Borate.

Fig. 5. The aerobic oxidation of *M/120* formate in atmospheric oxygen at 16° and 40°. Ordinate: velocity, being difference between oxygen uptakes in the readings at 15 and 30 minutes. Abscissa: p_H . Bacterial nitrogen for each experiment at 40° 0.6 mg.; at 16° 1.2 mg.

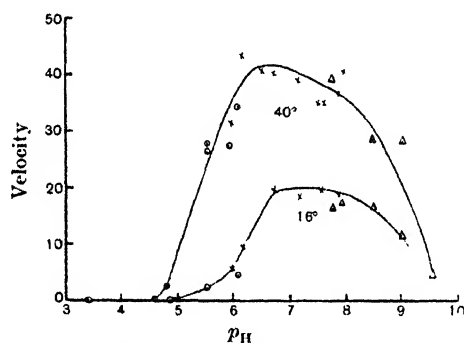


Fig. 6. The oxidation of *M/120* lactate at 16° and 40°.

Ordinate and abscissa as for Fig. 5. Bacterial nitrogen at both temperatures 1.2 mg.

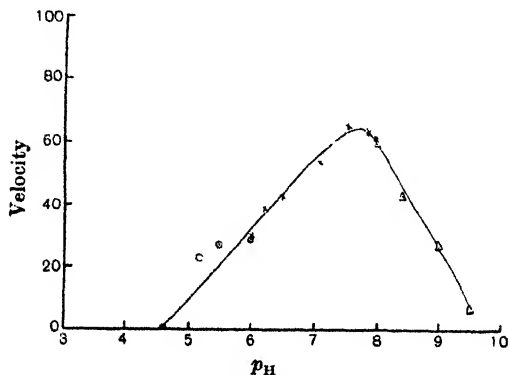


Fig. 7. The oxidation of *M/120* succinate at 40°. Bacterial nitrogen 1.2 mg.

treated organism was prepared and divided into 10 cc. samples. To *A* were added 20 cc. of Ringer's solution; to *B* 20 cc. of phthalate buffer at p_H 3.2, the resultant p_H being 3.38; to *C* were added 20 cc. of phosphate buffer at p_H 7.2; to *D* 20 cc. of borate buffer at p_H 10, the resultant p_H being 9.7; *E* the residual 10 cc. served as a second control and was kept on ice. The suspensions were well mixed and allowed to stand at 18° for 1½ hours. The mixtures were then centrifuged and each sample made up to the original volume of 10 cc. The aerobic oxidation of *M*/120 formate at p_H 6.3, and of *M*/120 lactate and succinate at p_H 7.6 and 40° was then investigated. The methylene blue reductions were carried out with the same concentrations of substrates, but at p_H 7.6 in each case. The results are shown in Table I. In these experi-

Table I. *Percentage of original velocity remaining after exposure of the bacteria to the p_H shown for 1½ hours at 18°.*

Methylene blue reductions: p_H 7.6.

Oxygen reduction: succinate and lactate, p_H 7.6; formate, p_H 6.3.

Temperature 40°.

	Bacteria exposed to							
	<i>A</i>		<i>B</i>		<i>C</i>		<i>D</i>	
	Ringer		p_H 3.38		p_H 7.2		p_H 9.7	
	O ₂	M.b.	O ₂	M.b.	O ₂	M.b.	O ₂	M.b.
Formate	100	100	12.2*	56.5	72.7	100	36	80.5
Succinate	"	"	7.0	<3.0	86.3	95.7	58.6	48.3
Lactate	"	"	23.6	"	109	88.1	61.6	74

* Initial velocity about 60 % of original velocity.

ments a reduction of less than 15 % is not to be considered significant, as it is somewhat difficult to ensure the presence of equal amounts of bacteria in the several experiments. The results show that exposure to an acid p_H diminishes considerably the activity of lactic and succinic dehydrogenases, formic still retaining rather more than half its activity. At p_H 7 none of the dehydrogenases is appreciably affected, but at p_H 9.7 the dehydrogenases are affected in the order of activity succinic, lactic and formic. These results agree with those obtained by Quastel and Wooldridge [1927] for the untreated organism. In the aerobic experiments the effect of the acid p_H is most marked with succinate, this being followed by formate and lactate. At p_H 7.2 the oxidation of lactate and succinate is not appreciably affected, but that of formate is decreased by 23 %, while at a more alkaline p_H the effect is in the order formate, succinate, and lactate.

DISCUSSION.

The results obtained in the aerobic oxidations are more complicated in that they involve a second factor in the activation of the oxygen. An interpretation must first be given to the anaerobic oxidations in which it is clear that dehydrogenation of the substrate is the main factor concerned. A glance

at Figs. 2, 3 and 4 will show that the p_H -activity curves of the dehydrogenases are of quite a different type from those obtained with hydrolytic enzymes.

Michaelis and Davidsohn [1911] suggested from the results obtained on the p_H -activity curve of invertase that the enzyme was an amphoteric electrolyte. This conception has been extended by Woolf [1931] who has based his conclusions on the results obtained with fumarase [Mann and Woolf, 1930]. Woolf points out that the symmetrical p_H -activity curve obtained for hydrating enzymes is not given by dehydrogenases, and suggests that the curves represent the action of the enzyme as an ionised acid or an un-ionised base. The curves shown in Figs. 2, 3 and 4 therefore represent the dissociation curve of an acid or the dissociation residue curve of a base. But the curves obtained, especially those in Fig. 2, show deviations from this ideal form. A further study of the formate oxidation is in progress. The curves obtained for lactate and succinate are of the same type as that obtained for formate, although the point at which the curve reaches the plateau is different with the different substrates (Table II).

Table II.

	Formate	Lactate	Succinate
M.b. plateau starts (p_H)	7.0	7.4	8.5
O ₂ optimum (p_H)	6.0	6.3	7.6
p_K of acid	3.68	3.86	5.28

The figures obtained for the maximum velocity in oxygen will be referred to later. It will be seen from Table II that there appears to be some relation between the degree of dissociation of the acid concerned and the activity of the dehydrogenase. The p_K of succinic acid has been taken as that of the second ionisation. It may be postulated tentatively that the dehydrogenases are acting as un-ionised bases, the activity being dependent on combination with the fully dissociated acid. This active enzyme-substrate complex then breaks down, the hydrogen of the substrate being transferred to the methylene blue.

The curve obtained for succinate dehydrogenation is similar to that obtained by Ohlsson for extracted muscle succinoxidase. This writer also observed the inhibitory effect of the borate buffer, but continued his experiments in glycine-phosphate buffer. This buffer is not satisfactory for work with bacteria, as deamination may occur. The curve obtained by Quastel and Whetham for resting untreated *B. coli* rises to an optimum at about p_H 7.2 and continues as a plateau to p_H 10. No inhibitory effect is noted with borate buffer. These results may be explained by the fact that the p_H -activity curve of the toluene-treated organism resembles more closely that of an extracted enzyme. It is perhaps significant that the velocity of dehydrogenation is increased by about 30 % after toluene-treatment. In the aerobic experiments with formate and lactate there is no significant difference in the shape of the curves at 16° and 40°. It would therefore appear that when activation of oxygen is the limiting factor, hydrogen ion concentration does not alter the relation between

dehydrogenase and oxygenase. Figs. 5, 6 and 7 show that the curves obtained in some respects resemble those for the dehydrogenases, but that the oxygen optima have been shifted in relation to the edge of the methylene blue plateau to the acid side in all three cases. In Table II the oxygen optima are given and it will be seen that the shift is in each case equal to approximately one p_H unit. It will also be noted from the figures that at the point where a change is made from borate to phosphate buffer no appreciable change in velocity occurs, although there is a difference of about 40 % in velocity at this point in the anaerobic process. A similar effect has been noted by Cook, Haldane and Mapson [1931] with the action of 8-hydroxyquinolinesulphonic acid. From these experiments it is evident that for the aerobic oxidations the activity of the dehydrogenase is not always the limiting factor in the reaction. The shift of the p_H optima in the aerobic oxidations would perhaps indicate that it is the activity of the oxygenase that is limiting the reaction. It will also be seen from Table I that after exposure to p_H 3.38 although there is a considerable diminution in the activity of the lactic and succinic dehydrogenases there is still an appreciable oxygen uptake. With formate the initial velocity corresponds to about 60 % of the original velocity, but the value given represents the velocity when the system is in a steady state.

It is difficult to give any complete interpretation of the curves obtained in the aerobic experiments—as in all cases the process is complicated by the action of the dehydrogenase. A study of the oxidation of indophenol or some related compound in which activation of the oxygen is the sole process concerned would possibly throw some light on this process.

It is evident from these results that the process of oxidation is one of considerable complexity and that the study of the effect of hydrogen ion concentration on the oxidative mechanisms gives us one method of attack on the mechanism of correlation of the various enzymes concerned.

SUMMARY.

1. A study has been made of the effect of hydrogen ion concentration on the aerobic and anaerobic oxidations of formate, lactate and succinate by toluene-treated *B. coli*.

2. The anaerobic oxidations in the presence of methylene blue give p_H -activity curves which rise steadily from the acid region to a plateau which starts at about p_H 7.0 with formate, p_H 7.4 with lactate and p_H 8.5 with succinate. There is a steady drop from the plateau at about p_H 9.5 in all cases.

3. The p_H -activity curves obtained in the aerobic oxidation of the three substrates resemble in general shape the curves for the dehydrogenases, but the optima have been shifted to the acid side.

4. Exposure to solutions of low p_H inactivates the dehydrogenases in the order succinate, lactate and formate. The aerobic mechanism is affected about equally in each instance.

5. The effect is not so marked in the alkaline region. The oxidation of succinate both anaerobically and aerobically and the aerobic oxidation of formate are the mechanisms most affected.

It is with very great pleasure that we express our thanks to Prof. J. B. S. Haldane for many valuable suggestions and ever ready advice. We wish to thank Dr B. Woolf for allowing us to read his paper in proof. To Sir F. G. Hopkins we are indebted for interest and encouragement.

REFERENCES.

- Bernheim (1928). *Biochem. J.* **62**, 344.
Cook (1930). *Biochem. J.* **24**, 1538.
——— Haldane and Mapson (1931). *Biochem. J.* **25**, 534.
——— and Stephenson (1928). *Biochem. J.* **22**, 1368.
Dixon and Thurlow (1924). *Biochem. J.* **18**, 976.
Haldane (1930). *Enzymes* (Longmans, Green and Co., London).
Hare (1928). *Biochem. J.* **22**, 968.
Mann and Woolf (1930). *Biochem. J.* **24**, 427.
Michaelis and Davidsohn (1911). *Biochem. Z.* **35**, 386.
Ohlsson (1921). *Skand. Arch. Physiol.* **41**, 77.
Quastel and Whetham (1924). *Biochem. J.* **18**, 519.
——— and Wooldridge (1927). *Biochem. J.* **22**, 605.
Woolf (1931). *Biochem. J.* **25**, 342.

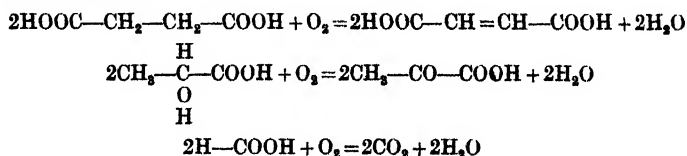
LXIII. THE RELATIONSHIP BETWEEN THE RESPIRATORY CATALYSTS OF *B. COLI*.

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(Received February 28th, 1931.)

DURING the process of respiration in the cell two different types of activation by catalysts have been described. On the one hand organic molecules are rendered more susceptible to oxidation, and on the other oxidising substances more prone to reduction. The first type of activation commonly leads to dehydrogenation in Wieland's sense, and is carried out by dehydrogenases. The activation of oxygen has been particularly stressed by Warburg [1926]. He describes the responsible catalyst as the *Atmungsferment*. As, however, we cannot agree with his view that it is the only catalyst concerned in respiration we prefer to employ the term oxygenase in accordance with the usual practice of naming an enzyme after its substrate. Bach's [1913] oxygenase is possibly not an oxygen activator, but an enzyme which, in conjunction with catechol or a related substance, reduces oxygen to hydrogen peroxide. The main object of the present research was to determine the relationship between these two types of activation. Whereas the dehydrogenations so far studied in living cells have usually been simple, the oxidations by molecular oxygen have almost always been complex. Thus the complete oxidation of glucose must involve very many consecutive reactions, and even that of alcohol or acetic acid more than one. Hence it is difficult to interpret the effects of heat, poisons, or other alteratives on such complex processes. Cook [1930] showed that after treatment with toluene-saturated water *B. coli* still performed the following oxidations:



The oxidation of formic acid proceeds to 98 % completion, those of succinic to fumaric, and of lactic to pyruvic acids proceed to about 82 % and 76 % respectively. In neither case does the oxidation proceed any further, no CO₂

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being produced in the latter two. The rates do not differ greatly from those in the untreated organism.

The toluene-treated organism has the further advantage that it has no metabolism in the absence of added substrate. Not only does it not consume O_2 but it does not reduce methylene blue *in vacuo* even after several days.

The bacteria used were a strain (N.T.C.) from the National Type Culture collection, and a strain (O.P.S.) from the University Pathological Department, suspensions being made as described by Cook and Stephenson [1928], with frog Ringer's solution. These were diluted with water so as to give a suitable rate of reduction of oxygen or methylene blue. Fuller details are given by Cook and Alcock [1931]. Carbon monoxide was obtained from a cylinder. It contained small amounts of nitrogen and hydrogen as impurities, as well as CO_2 , which was absorbed in consequence of the technique used.

OBSERVATIONS ON THE DEHYDROGENASES OF THE TOLUENE-TREATED ORGANISM.

The properties of succinic, lactic, and formic dehydrogenases have been studied by Quastel and his colleagues from 1924 onwards and by Cook [1930], but certain further observations were required. The Thunberg vacuum tubes used contained 2 cc. 0.2 *M* phosphate buffer, 1 cc. 0.002 *M* methylene blue, 0.5 cc. 0.2 *M* substrate, and 1.5 cc. H_2O , to which inhibitors were added as required. These were brought to the required p_H with H_3PO_4 or NaOH.

Finally, 1 cc. of bacterial suspension diluted with water was added. The substrate concentration was thus *M*/120. The tubes were evacuated for 1 minute and then placed in a bath at 40°. The time taken for 90 % reduction was measured. All measurements were made at least in duplicate.

Effects of cyanide.

The effects of KCN on the rate of methylene blue reduction are shown in Fig. 1. The ordinates are the reciprocals of the reduction times. It will be seen that there is a moderate activation by low concentrations of cyanide, which is clearly significant in the case of succinic dehydrogenase. The greater effect of cyanide on formic dehydrogenase at the more acid p_H is doubtless due to the greater amount of HCN in equilibrium with cyanide ions at that p_H . The irregularities are perhaps due to the fact that variable amounts of HCN were pumped off in different experiments. It will be seen that there is no very serious inactivation of any of the dehydrogenases below a concentration of 0.002 *M*, whereas catalase and peroxidase are largely inactivated by concentrations of 10^{-5} *M*. Hence any marked effects of cyanide concentrations below 0.002 *M*, such as are shown in Fig. 3 are not due to effects on the dehydrogenases. No reduction of methylene blue occurs in presence of cyanide and buffer alone.

Effects of carbon monoxide.

In order to study the effect of CO this gas was passed through alkaline pyrogallol solutions to absorb O_2 and CO_2 . The substrate, dissolved in only 0.1 cc. of water, was placed in the small limb of the vacuum tube, which was evacuated; CO was run in, the tube re-evacuated and more CO added. It was then cooled on ice, shaken vigorously for exactly 3 minutes, and placed in a thermostat. Controls were filled in just the same way with N_2 . CO has no

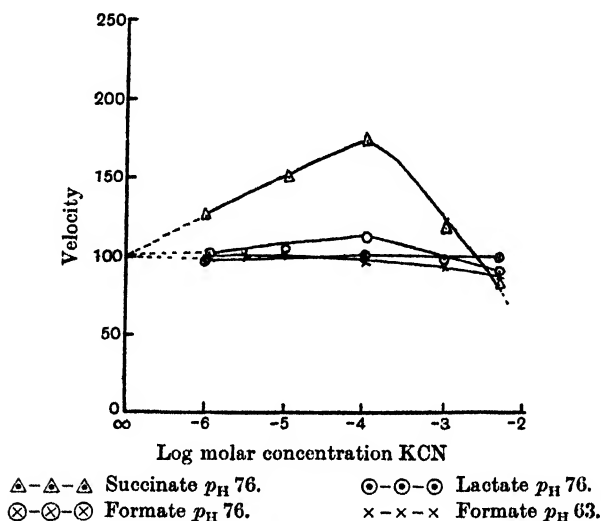


Fig. 1. Effect of KCN on rates of methylene blue reduction.

effect on lactic dehydrogenase at 40° . At 25° it seems to have a small effect on formic dehydrogenase. Thus at 25° the reduction times in N_2 were 4.15, 4.25, 4.25, in CO 5.15, 5.45. However, at 16° there is no such effect. Thus reduction times in N_2 were 17.00, 17.10 and 16.45 minutes, in CO 16.40, 16.55 and 16.50. As the experiments on oxygen uptake in presence of CO were almost all made at this temperature, it is clear that the dehydrogenases were not being poisoned in them.

The Hecht-Eichholtz reagents and phenylurethane.

Hecht and Eichholtz [1929] and Eichholtz [1929] introduced the use of a system of organic compounds as heavy metal reagents in biochemical research. They form complexes with heavy metals, and the inhibition or acceleration [Krah, 1930] of a reaction is taken to show that the catalyst concerned contains a metal which combines with the reagent added. We have used the reagents 8-hydroxyquinolinesulphonic acid (Q), 2-aminophenol-4-sulphonic acid (A), and 1-amino-8-naphthol-4-sulphonic acid (N) at 40° . A has no marked effect on any of the dehydrogenases. Q has no effect on reduction by lactates. In very high concentrations such as 0.4 % Q slightly accelerates

reduction by succinate, the velocity being increased by 21 %. Q has a large effect on formate, as shown in Fig. 2. The effect is greater at p_H 6.3 than at 7.6, as with the acidic poisons used by Myrbäck [1926]. N has a very great inhibitory effect on reduction by formate. These results suggest that formic dehydrogenase contains copper and not iron. For Q and N, which inhibit, both

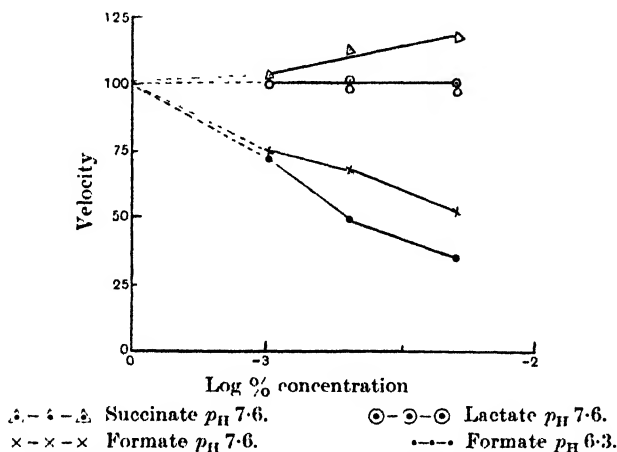


Fig. 2. Effect of 8-hydroxyquinolinesulphonic acid on rates of methylene blue reduction.

unite with copper. Q also unites with Mn, Co, and Ni, N also with Fe, while A unites with Fe only. Inhibition by Q is independent of variations in the concentration of methylene blue, and is no greater when the bacteria have stood with the reagent for $1\frac{1}{2}$ hours before the methylene blue is added.

No inhibition was obtained with phenylurethane on lactic or formic dehydrogenases.

EXPERIMENTS ON O_2 REDUCTION.

The Method

Oxygen uptakes were studied in Barcroft apparatus. Each cup contained 1.0 cc. of a suspension of the toluene-treated organism, 1.0 cc. of a 0.5 *M* buffer solution, 0.5 cc. of a donator solution for which distilled water was substituted in the left-hand pot, and 0.5 cc. of distilled water or solution of an inhibitor. The p_H was always 7.6 in the case of succinate and lactate, this hydrogen ion concentration being nearly optimal, as shown by Cook and Alcock [1931]. In the case of formate, the p_H was either 7.6 or more usually the optimal 6.3. From time to time the p_H after the end of the reaction was measured with a hydrogen electrode and was found to be unaltered in the case of lactate and succinate. Formate solutions became more alkaline by about 0.2 p_H at most. The substrate concentration was usually *M*/60, as at this concentration the enzymes concerned are saturated with their substrates, and the rate of oxidation does not therefore fall off with a moderate diminution in the substrate concentration. Gas mixtures were made up and stored over water in aspirators. They were analysed with the Haldane apparatus.

To introduce them into the Barcroft apparatus the right-hand tube of the latter is connected to the aspirator with rubber tubing. The apparatus is then brought nearly into thermal equilibrium with the thermostat. The left-hand pot is left in position, the right-hand pot held so that a small crevice is left between it and its stopper. The gas is then introduced, the pot being held so close that there is always a positive pressure within it, as shown by the manometer. About half a litre is then run through, the pot pushed home and the tap closed. After 5 minutes' shaking in the bath the taps are opened to level the oil in the manometer, and the experiment begins. Analyses of gas from the pot agreed with that introduced to within 0.3 %. In a few experiments the manometers were evacuated, and both cups filled with gas mixture. On repeating the process several times the air is pretty completely replaced by gas mixture. The small central pot contained a roll of filter paper soaked in KOH to absorb CO_2 [Dixon and Elliott, 1930]. The apparent rates of oxygen uptake often did not become steady until half an hour had elapsed, due in part to the gases going into solution (see Fig. 5). The data here given refer to the linear parts of the curves. All experiments, including controls, were done at least in duplicate.

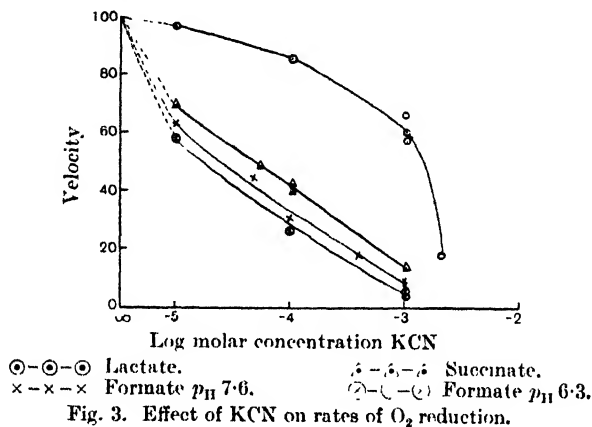
In the case of formate and lactate the oxygen uptake remained fairly constant from day to day. There were, however, occasional fluctuations, generally decreases in oxidising power on keeping. Hence we have not given full weight to experiments in which the effect of a poison was not measured simultaneously with a normal control, although when controls were done on the same day the error is likely to be small. In the case of succinate the oxidising power often fell off in the first few days of keeping a bacterial suspension. The power of reducing methylene blue sometimes fell off simultaneously; but often it remained normal when the oxygen uptake had dropped to less than 10 % of the original value. Hence our data on succinate oxidation are rather incomplete.

Effect of cyanide on oxygen uptake.

Precise measurements of the effect of cyanide on oxygen uptake are difficult, because the velocity of uptake, when inhibition is considerable, sometimes tends to fall off slightly with time. On the other hand, there is no question whatever as to the difference in the susceptibility of lactic dehydrogenase on the one hand, and succinic and formic on the other. This is at once clear from Fig. 3. The difference between the latter two is not so clearly significant, and may be due to the stimulating effect of cyanide on succinic dehydrogenase. As the dehydrogenases are hardly inhibited in concentrations below $10^{-3} M$ it is clear that the main effect here studied is a direct effect either on the oxygenase or on some intermediate substance. The dissociation constant of the oxygenase-cyanide compound (if we regard the effect as due to combination) would seem to be about $1.5 \times 10^{-3} M$ in the case of lactate, $5 \times 10^{-5} M$ in that of succinate, and $2 \times 10^{-5} M$ in that of formate.

Effect of phenylurethane on oxygen uptake.

Phenylurethane had a slight inhibitory effect on oxygen reduction by lactate and formate at 16°. The former process was reduced to 78 % of its original velocity by 0.5 cc. saturated phenylurethane solution in the total volume of 3 cc. Formate oxidation was reduced to 75 % by saturated phenylurethane solution and to 83 % by *M/6000* solution.



When, however, the oxidation had already been partly inhibited by cyanide, saturated phenylurethane was more effective. The uptake of lactate in presence of $10^{-3} M$ KCN was reduced to 24 % of its value with KCN alone, that of formate in presence of $10^{-5} M$ KCN to 56 % of its former value.

Effect of carbon monoxide.

A large number of experiments have been done in mixtures containing varying amounts of CO, O₂ and N₂. As a preliminary it was shown that the

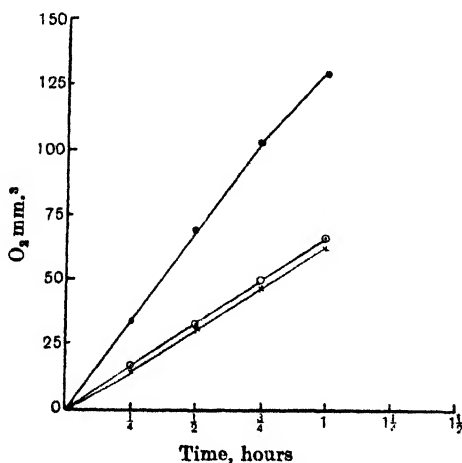
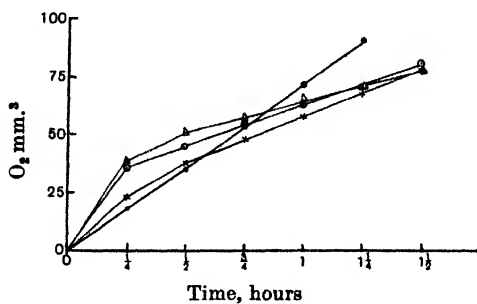


Fig. 4. Oxygen uptakes at 16° and p_H 6.3 in presence of formate from air and two mixtures containing different amounts of CO, but similar CO/O₂ ratios.

oxygen uptake remains unaltered when air is replaced by pure O_2 , or diluted with N_2 so that the partial pressure of O_2 is reduced to 5 %. Hence neither diffusion of O_2 nor incomplete saturation of oxygenase with it was a limiting factor. Several experiments, of which Fig. 4 is typical, showed that as found by Warburg [1926] the inhibition by CO depends solely on the ratio of CO to O_2 , and not on the partial pressure of CO. It at once becomes clear that the oxidation of formate is more sensitive to CO than that of succinate or lactate. If V be the velocity of O_2 uptake in absence of CO, v that in its



--- Air. $\times-\times-\times$ CO/O_2 4:1. $\odot-\odot-\odot$ CO/O_2 7:1. $\triangle-\triangle-\triangle$ CO/O_2 13:6.

Fig. 5. Oxygen uptakes at 16° and p_H 7.6 in presence of succinate from air and various gas mixtures containing CO.

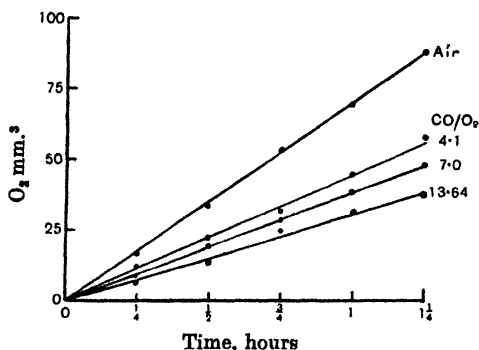


Fig. 5 a. Data of Fig. 5 plotted after the first half-hour.

presence, r the ratio of partial pressures of CO to O_2 , and K the affinity of oxygenase for oxygen divided by its affinity for CO, then Warburg shows that $v = \frac{KV}{K+r}$, or $K = \frac{r}{\frac{v}{V} - 1}$, provided certain assumptions examined later are made.

From such data as those of Figs. 5, 6 and 7 it is possible to calculate values of K . Unfortunately the majority of our data are dubious for one of four reasons. Duplicates do not agree, uptakes are not linear, experiments with and without CO were not done simultaneously, or the temperature had varied. The results vitiated in any of these ways are grouped as second-class results in Table I. No results whatever have been rejected.

Details of a typical experiment are given below.

Two manometers containing N.T.C. bacilli and *M/60* formate at 16° and p_H 6.3 gave uptakes of 76.6 and 78.9 mm.³ O₂ in the half-hour between $\frac{1}{2}$ an hour and 1 hour after the beginning of the experiment. Two others containing the above in presence of 59.9 % CO and 7.49 % O₂ gave uptakes of 56.6 and 60.8 mm.³ O₂ in the 2 hours from $\frac{3}{4}$ hour to $2\frac{3}{4}$ hour after the beginning. In each case the O₂ uptake was linear over this period. The mean uptakes per hour were therefore 155.5 mm.³, and 29.34 mm.³ The velocity was thus reduced to 18.9 %, giving a *K* value of 1.86, with an uncertainty of ± 0.11 , allowing for the differences between duplicate oxygen uptakes and CO estimations.

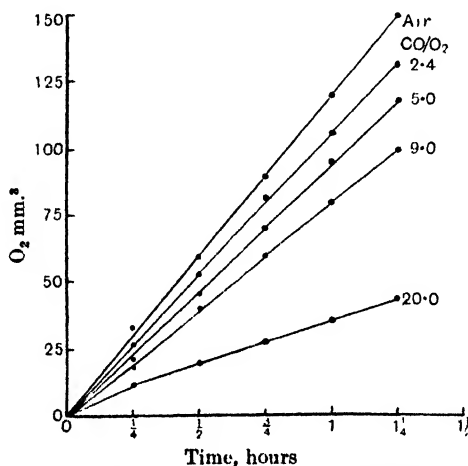


Fig. 6. Oxygen uptakes at 16° and p_H 7.6 in presence of lactate from air and various mixtures containing CO.

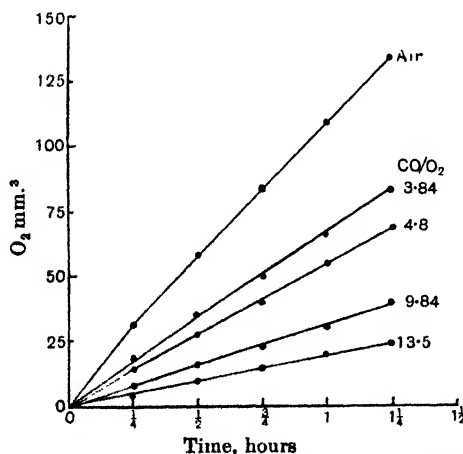


Fig. 7. Oxygen uptakes at 16° and p_H 6.3 in presence of formate from air and various gas mixtures containing CO (O.P.S. bacilli).

On the other hand, in some of the second-class experiments duplicates disagreed very markedly. Thus in the lactate experiment giving a *K* value of 17.4 the duplicate CO uptakes were 21.1 and 27.7 mm.³ The lower value, which was probably correct, would have given $K = 11.1$.

It will be seen that the two strains used differed considerably as regards the sensitivity of formate oxidation to CO. There were no significant differences as regards succinate and lactate oxidation. The difference between the two strains possibly arose during the experiments. Two second-class experiments on the N.T.C. strain in July 1930 gave values of 4.15 and 3.20 for K , while the other experiments at p_H 6.3 from October onwards gave values of 2.35 or lower. There can be no serious doubts as to the significance of the differences of the K values found. It will be observed that there is no overlapping among the first-class values. The second-class values do not show any certain difference between succinate and lactate, but both these differ clearly from formate.

Table I.

Values of K (ratio of CO to O₂ giving 50 % inhibition) at 16°.

Substrate and race	First-class results	Median	Mean	Second-class results	Median
Succinate	5.9, 6.8	6.3	6.3	5.0, 5.0, 6.0, 10.3, 13.8, 16.5	8.2
Lactate	8.2, 9.5, 9.6, 9.7, 11.8, 12.2, 15.7*	9.7	10.1	3.8, 6.5, 6.9, 7.4, 7.8, 8.7, 10.2, 11.0, 13.4, 14.4, 16.4, 17.4	9.5
Formate (O.P.S.)	3.39, 3.45, 3.54, 4.14	3.49	3.62	1.75, 3.14, 3.45, 3.88, 4.46	3.45
Formate (N.T.C.)	1.86, 2.27, 2.35, 1.40†	2.27	2.15	0.94, 1.07*, 1.29, 1.67, 2.30, 2.95‡, 3.20, 4.15	1.98

* At 18°.

† At p_H 6.0.‡ At p_H 7.6.

In Fig. 8 the observed velocities for lactate oxidation in presence of CO, as percentages of those in its absence, are plotted against the CO/O₂ ratio.

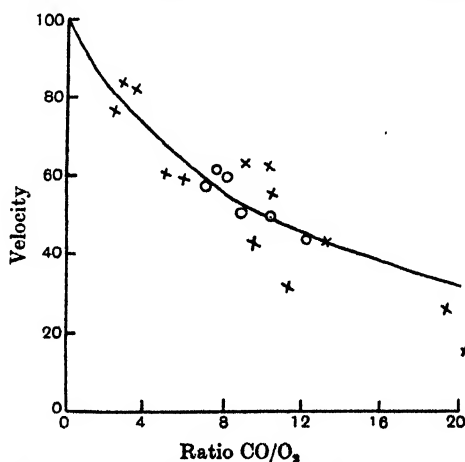


Fig. 8. Velocities of oxygen uptake (as percentages of that in air) at 16° and p_H 7.6 in presence of lactate from gas mixtures containing CO and O₂ in different ratios. The curve represents velocities calculated for $K=10$.

There is no decided evidence for a significant departure from a rectangular hyperbola, such as Warburg [1926] found in the case of yeast. The two velocities plotted on the right in Fig. 8 are certainly low, corresponding to low

values of K , but neither is reliable. On the other hand, in the case of formate oxidation by O.P.S. bacilli the two lowest velocities, obtained with CO/O_2 ratios of 13.6 and 13.9, give a first-class K of 3.39, and a second-class K of 3.20 respectively. Both the velocities lay between 18 % and 19 % of that in air.

At 40° the effect of CO was much less (Fig. 9). With formate we obtained a first-class value of 9.8, with the O.P.S. strain, and second-class values of

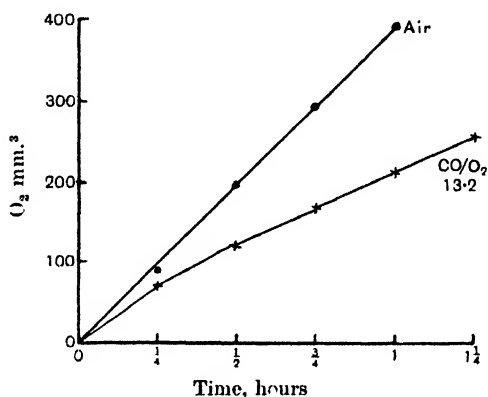


Fig. 9. Oxygen uptakes at 40° and $p_{\text{H}} 6.3$ in presence of formate from air and a gas mixture containing CO (O.P.S. bacilli).

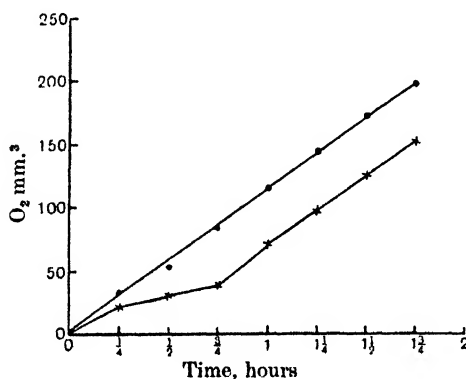


Fig. 10. Oxygen uptakes at 16° and $p_{\text{H}} 7.40$ in presence of formate from a gas mixture containing 65 % CO and 7.7 % O_2 . After 45 minutes this was replaced by air.

40, 9.7, 6.7, and 5.7 with the N.T.C. strain. The effect on lactate oxidation was negligible with a CO/O_2 ratio of 13.2, which would have given 59 % inhibition at 16° . The value of K is probably at least 50.

Inhibition by CO is completely reversible (Fig. 10). Experiments carried out on bacteria exposed to CO in the strongest light at our disposal appeared to show an acceleration of about 5 % as compared with nearly complete darkness. The effect was, however, too small to measure accurately.

Combined effect of cyanide and carbon monoxide.

Four experiments were carried out to see what further inhibition was produced by CO on oxygen uptake already partly inhibited by cyanide. In two second-class experiments with lactate in presence of 0.001 *M* KCN we obtained values of 9.3 and 14.4 for *K*. With formate in presence of 0.001 *M* KCN we obtained, with N.T.C. bacilli, a first-class value of 2.16, and a second-class value of 2.47. Thus in presence of cyanide concentrations which themselves produce an inhibition of 37 % and 70 %, the value of *K* was, if anything, slightly increased, *i.e.* the system rendered less sensitive to CO.

Combined effect of oxalate and carbon monoxide.

As a preliminary to the use of oxalate it was shown that the values of *K* are unaltered by washing the bacilli free from calcium.

Oxalate inhibits the reduction of methylene blue by lactate, and has a similar effect on oxygen reduction. The latter is not, however, quite so great as the former. Thus in one series of experiments performed simultaneously the velocity of methylene blue reduction was reduced to 39.7 ± 2.3 %, that of oxygen reduction to 52.6 ± 1.9 %, the errors being the extreme values calculable from the observations.

Oxalate has no marked effect on O₂ reduction by formate [Cook, 1930] nor on the *K* value of this process. With N.T.C. bacilli we obtained a first-class value of 2.30 and a second-class value of 1.29 in 0.0027 *M* oxalate, in 0.0017 *M* oxalate a second-class value of 1.63. In 0.0076 *M* oxalate a first-class experiment gave *K* = 1.86. Thus, if anything, oxalate makes formate oxidation more sensitive to CO, but the effect, if any, is within the limits of experimental error.

On the other hand, oxalate renders the oxidation of lactic acid far less sensitive to CO. In a concentration of *M*/3000 we obtained a first-class value of *K* of 19.0, and second-class values of 42 and 468, which at least demonstrate that there was hardly any inhibition.

The combined effect of phenylurethane and carbon monoxide.

Although, as pointed out above, phenylurethane has only a slight effect on oxygen reduction, it renders the system extremely sensitive to CO. Thus the value of *K* for lactate oxidation in presence of 0.00011 *M* phenylurethane fell to 0.86 in one first-class experiment, that for formate oxidation by N.T.C. bacilli fell to 0.24 and 0.17 in two good second-class experiments. Clearly the ratio of the affinities of the oxygenase for CO and O₂ has been increased about tenfold. It will be remembered that phenylurethane also renders the system more sensitive to cyanide, though in this case the effect is less striking.

The effect of 8-hydroxyquinolinesulphonic acid.

In spite of its marked inhibitory effect on methylene blue reduction by formate, 8-hydroxyquinolinesulphonic acid has no effect on oxygen reduction by this substrate. Thus a 0.004 *M* concentration at 16° gave a 2 % reduction in oxygen uptake by formate, and a 1 % increase in that by lactate, both well within the limits of experimental errors. Nor is there any definite effect on the value of *K* in the case of formate. A second-class experiment on N.T.C. bacilli gave *K* = 1.68.

Experiments with mixtures of formate and lactate.

Cook [1930] showed that at 40° the oxygen uptake in presence of lactate and formate is equal to the sum of those in presence of either alone. This was found to be true at 16° also. Thus a suspension absorbed 64.3 mm.³ of O₂ in half an hour in presence of formate, 39.2 in presence of lactate, while in a mixture the quantity was 103.2 mm.³, the calculated value being 103.5. This mixture was now exposed to CO and gave a value of *K* of 1.66. The data are of the second-class, owing to disagreement of duplicates in presence of CO, and the true value was probably higher.

Oxygen uptakes in presence of methylene blue.

A series of experiments was carried out to see how far oxygenase can be replaced by methylene blue. Oxygen uptakes were measured alone, in presence of added methylene blue, of added cyanide and of methylene blue and cyanide. Fig. 11 shows that in the case of formate when the oxygenase has

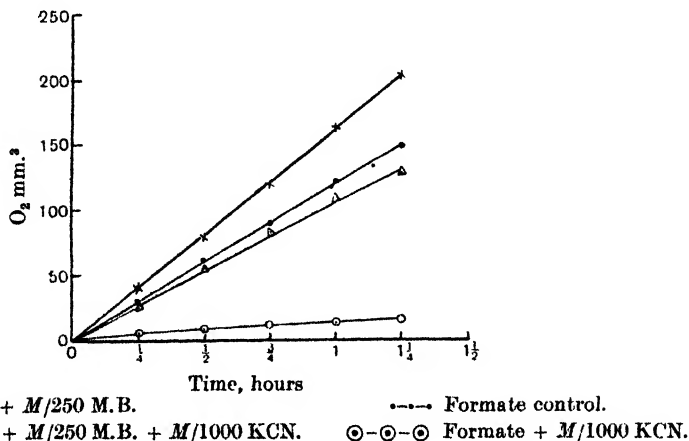


Fig. 11. Oxygen uptakes from air at 16° and p_{H} 6.3 in presence of formate alone, with the addition of KCN, of methylene blue and of both.

been mainly poisoned by cyanide, it can be replaced to a considerable extent by methylene blue. This is reduced by the formic dehydrogenase and re-oxidised by air, this re-oxidation not being prevented by the cyanide. Fleisch

[1924] and Stephenson [1928] have obtained similar results. In other experiments the uptake in presence of formate has been actually increased by the addition of KCN and methylene blue. Thus at p_H 7.6 the oxygen consumption in the same period of half an hour was 47.0 mm.³ in the absence of cyanide and methylene blue, and 63.6 when both were present. Duplicates agreed well.

The oxidation of lactate was affected differently. We were never able to get more than 67 % of the original oxidation on adding methylene blue and cyanide, and methylene blue alone reduced the rate of uptake to about 74 % of the normal value. We have no satisfactory results in the case of succinate at 16°. At 40° methylene blue causes a slight acceleration.

The system in which, in presence of cyanide, oxygenase is replaced by methylene blue can be used as a model for the action of CO on the oxygenase. When such a system is exposed to air containing CO the rate of oxygen uptake falls off rather slowly, and finally may settle down to a definite velocity. In the steady state the methylene blue can be seen to be mainly reduced. This is explained by the discovery of Reid [1930] that the re-oxidation of leuco-methylene blue is a metal catalysis inhibited by CO. Presumably the KCN and CO between them unite with almost all the catalytically active metal. The degree of inhibition depends on the partial pressure of O₂ as well as that of CO. Thus in presence of formate at p_H 6.3 we obtained an uptake of 11.4 mm.³ per hour in presence of 52.5 % CO and 9.68 % O₂, but of 50.8 mm.³ in presence of 52.5 % CO and 46.3 % O₂. It had previously been shown that, in the absence of CO, the rate of oxygen uptake did not depend on the partial pressure of O₂. In another set of four experiments the rate was shown to be the same when the ratio of CO to O₂ was kept constant, the absolute amount being altered. The value of K was in one case 0.18, but it varied in different experiments, probably because the amount of catalytically active heavy metal was not constant.

DISCUSSION.

The experiments on methylene blue reduction can be interpreted without difficulty. The stimulating effect of small amounts of cyanide can probably be explained by its combination with small amounts of heavy metal which inhibit succinic dehydrogenase, as shown by Quastel and Wooldridge [1927]. A similar explanation of their activating effect on proteinases has been given by Krebs [1930]. The inhibition by relatively high concentrations of cyanide may be due to its union with heavy metals forming part of the enzymes. But the Hecht-Eichholz reagents give no indication of metal in succinic and lactic dehydrogenases, though they point strongly to the presence of copper in formic dehydrogenase and suggest that copper is inhibiting succinic dehydrogenase.

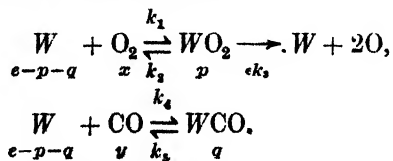
Cook [1930] showed, by the specific effect of malonate on the oxidation of succinate by methylene blue and oxygen, that the same catalyst was probably activating the succinate in each case, *i.e.* that succinate is activated by succino-dehydrogenase before it reduces oxygen. The same is true for lactate. But

this does not hold for formate, as 8-hydroxyquinolinesulphonic acid inhibits the reduction of methylene blue, but not of oxygen. And whereas methylene blue does not fully replace oxygenase in the oxidation of lactate, it more than does so in the case of formate. The suggestion is obvious either that oxygenase is the limiting factor throughout, or that the formate-activating mechanism which is inhibited by 8-hydroxyquinolinesulphonic acid is not normally concerned in oxygen reduction, but is made available for this process by the addition of methylene blue. The work of Stephenson and Stickland [1931] suggests that it may be related to hydrogenase in the same way as lactic dehydrogenase is related to oxygenase. The complexity of the curve relating oxygen reduction by formate to p_H [Cook and Alcock, 1931] suggests that the system concerned is particularly complex.

The fact that CO inhibits O_2 reduction in a competitive manner makes it quite clear that it acts on an activator of O_2 , as Warburg has pointed out. On the other hand, the difference between the apparent relative affinities of oxygenase in the three different oxidations studied suggests strongly that we are dealing with three oxygenases which differ from one another in this respect as do the haemoglobins of different species and individuals [Douglas, Haldane and Haldane, 1912; Anson *et al.*, 1924]. Further the relative affinities of the oxygenases concerned in formate oxidation in two different races of *B. coli* differ in this manner. Evidence for the existence of two oxygenases in *Galleria mellonella* which differ in their relative affinities has already been given by Haldane [1927].

It is clear, moreover, that, even if we do not accept the above theory, the actual molecules of oxygenase concerned in formate and lactate oxidation are different. For not only are the oxygen uptakes additive on mixing under normal conditions, but this is still true when they have been reduced by cyanide, so that oxygen activation is clearly the limiting factor. Thus in presence of 10^{-4} M KCN the oxygen uptake by formate was 70 mm.³ per hour, by lactate 80, by the two together 150. It therefore seems to be impossible that the three dehydrogenases should draw on the same common stock of oxygenase, even if the oxygenase associated with each is of the same molecular species.

Warburg [1926] believes that differences in the apparent affinity ratio K can be reconciled with the existence of only one oxygenase, on the following theory, re-stated in the terminology of Briggs and Haldane [1925] and Wurmser [1930]. Consider the reactions:



Where W represents oxygenase, and the molecular concentrations of the reactants and the velocity constants are as above. ϵk_3 is the constant of the

reaction actually measured and is supposed to be independent of the concentration of reducing substrate when this reaches a certain value, but to fall off when this is lowered, as in sugar-free buffer solution, ϵ being reduced from unity to a fraction.

Then the velocity of oxygen uptake is $\epsilon k_3 p$, or

$$\frac{\epsilon k_3 e x}{x + \frac{(k_2 + \epsilon k_3)}{k_1} \left(1 + \frac{k_4 y}{k_5}\right)}.$$

But x is very large compared with $\frac{k_2 + \epsilon k_3}{k_1}$, since if $y = 0$, the velocity is independent of x ; hence the velocity is

$$\frac{\epsilon k_3 e}{1 + \frac{(k_2 + \epsilon k_3) k_4 y}{k_1 k_5}}.$$

Hence $K = \frac{k_1 k_5}{(k_2 + \epsilon k_3) k_4}$, and therefore lies between $\frac{k_1 k_5}{(k_2 + k_3) k_4}$ and $\frac{k_1 k_5}{k_2 k_4}$.

If k_2 is small compared with k_3 , K may become very large when ϵ is small. The fact that the value of K for lactate oxidation rose when the lactic dehydrogenase was inhibited by oxalate completely confirms Warburg's theory. But it is also clear that the value of K can be altered by other means. For phenylurethane does not greatly reduce the velocity of oxygen uptake in the absence of CO, and hence cannot greatly change ϵ . But yet it has a very large effect in lowering the value of K .

On the theory that there is only one oxygenase, or *Atmungsferment*, we should have to suppose that whereas most of the oxygenase concerned in formate oxidation is in use, only a fraction is active in the case of succinate, and a still smaller fraction in that of lactate. In this case, however, KCN, by inhibiting part of the oxygenase, should diminish the value of K . It does not. The order of susceptibilities to KCN is the same as that to CO, but in this case the apparent affinities are nearly the same for formate and succinate oxidation, both being very much greater than for lactate oxidation. In the case of CO formate is far more susceptible than succinate or lactate. Moreover, whereas the oxygenases associated with lactate and formate oxidation are very stable, that concerned with succinate is unstable, even when the dehydrogenase is unaltered. Again it is known that the oxygenases of different organisms differ widely in the susceptibilities of their CO compounds to light [Keilin, 1929] and that this difference does not run parallel with their apparent affinities to CO, as it should if ϵ is the only variable concerned. Hence without ruling out the possibility of an explanation on Warburg's lines, we regard it as probable that there are at least three different kinds of oxygenase in *B. coli*. They probably all possess the same prosthetic group, but differ, as do haemoglobins, in the molecule to which it is attached.

We find that the inhibition by CO falls off very strongly with temperature. Warburg [1926] found no definite temperature effect, and Keilin [1929] found that in the case of yeast a rise of temperature increased the inhibitory effect of CO on oxygen consumption while having no effect on CO inhibition of

indophenol oxidase. On the other hand, the inhibition by CO of oxygen uptake by heart muscle and potato catechol oxidases showed a falling off with rising temperature, as in our case. We believe that this is due to a real diminution in the ratio of the affinities for CO and O₂, because there is a strong reason to think that oxygen activation is a limiting factor at 40°, even if it is not so at 16° [Cook, 1930]. Thus the majority of cases show a rise of *K* with temperature. The corresponding quantity for a human haemoglobin rises from 0.0025 at 15° to 0.004 at 37° [Anson *et al.*, 1924]. (Warburg's *K* is the reciprocal of Barcroft's.)

Finally we have to consider the relation between a dehydrogenase and the oxygenase associated with it. Three possibilities are open. Intermediate "carriers," such as cytochrome, glutathione, or hexuronic acid may diffuse from one to the other more or less freely. One or more molecules of each may be associated in a very small section of the cell, a minute reaction vessel, so to speak. Or the dehydrogenase and oxygenase molecules may be actually in contact. Our results are strongly against the first hypothesis, for we have shown that there is not a common stock of oxygenase, and on the whole in favour of the third. If it is true we can expect both oxygenase and dehydrogenase activity to be limiting factors simultaneously, as, for example, *p_H* and substrate concentration are in the case of a simple enzyme. Otherwise it is difficult to see why at 16° both CO and oxalate should give approximately hyperbolic curves when inhibiting lactate oxidation. If there were, for example, an excess of oxygenase, we should expect to get no effect from CO until a large amount of the oxygenase was poisoned. But we obtained 25.6 % inhibition of lactate oxidation with a ratio of CO to O₂ of only 2.4, the calculated inhibition being 19 %. For this reason an actual juxtaposition of the two catalysts seems plausible, though far from certain. Case [1931] comes to a similar conclusion regarding two muscle enzymes.

The results obtained must not be regarded as a picture of the mechanism of respiration in a typical cell. *B. coli* is a facultative anaerobe, its dehydrogenases are almost if not quite confined to its surface, and Dr Keilin kindly allows us to state that he finds it to contain cytochrome B and protohaematin, but has been unable to detect cytochrome A or C. A more normal type of cell might behave quite differently.

We have to thank Sir F. G. Hopkins for his great interest in our work, and Mr Meldrum for a valuable criticism.

SUMMARY.

1. We have studied the oxidation of succinate to fumarate, lactate to pyruvate, and formate to bicarbonate by the toluene-treated *Bacillus coli*. These reactions can each be studied uncomplicated by others.
2. Oxidation by methylene blue is little affected by KCN or CO, but the effects of other poisons suggest that formic dehydrogenase contains copper.

3. Oxidation by O_2 is inhibited by KCN and CO. The activators of oxygen concerned in the three catalyses differ in their susceptibilities to these poisons, and are regarded as differing as do different haemoglobins.

4. Phenylurethane has little direct action on these catalysts, but makes them more sensitive to CO and KCN.

5. Conclusions are drawn as to the way in which the various catalysts which are concerned in respiration co-operate with one another.

6. In presence of cyanide methylene blue can replace the activators of oxygen. Like theirs, its action is inhibited by CO.

REFERENCES.

- Anson, Barcroft, Mirsky and Oinuma (1924). *Proc. Roy. Soc. Lond. B* **97**, 61.
 Bach (1913). *Handbuch der Biochemie, Ergebnissband.* (Jena.)
 Briggs and Haldane (1925). *Biochem. J.* **19**, 338.
 Case (1931). *Biochem. J.* **25**, 561.
 Cook (1930). *Biochem. J.* **24**, 1538.
 — and Alcock (1931). *Biochem. J.* **25**, 523.
 — and Stephenson (1928). *Biochem. J.* **22**, 1368.
 Dixon and Elliott (1930). *Biochem. J.* **24**, 820.
 Douglas, Haldane and Haldane (1912). *J. Physiol.* **44**, 275.
 Eichholtz (1929). *Arch. exp. Path. Phar.* **148**, 369.
 Fleisch (1924). *Biochem. J.* **18**, 294.
 Haldane (1927). *Biochem. J.* **21**, 1068.
 Hecht and Eichholtz (1929). *Biochem. Z.* **206**, 282.
 Keilin (1929). *Proc. Roy. Soc. Lond. B* **104**, 206.
 Krah (1930). *Biochem. Z.* **219**, 443.
 Krebs (1930). *Biochem. Z.* **220**, 289.
 Myrbäck (1926). *Z. physiol. Chem.* **519**, 1.
 Quastel and Wooldridge (1927). *Biochem. J.* **21**, 224.
 Reid (1930). *Ber. deutsch. chem. Ges.* **63**, 1920.
 Stephenson (1928). *Biochem. J.* **22**, 606.
 — and Stickland (1931). *Biochem. J.* **25**, 205.
 Warburg (1926). *Biochem. Z.* **177**, 471.
 Wurmser (1930). *Oxydations et réductions.* (Paris.)

LXIV. CASEINOGEN FOR VITAMIN TESTS.

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THE basal diet used in vitamin A testing should, when supplemented by cod-liver oil, allow growth to maturity at what may be considered a normal rate; and the mean growth response of groups of animals should be graded to the dose of cod-liver oil given. If growth is restricted by the lack of some factor other than vitamin A, the result, at the best, may show such small differences in response to different doses of cod-liver oil as to make the significance of the differences doubtful. At the worst, the results may be so very irregular that no conclusion whatever can be drawn from them. It has recently been shown [Coward, Key, Dyer and Morgan, 1930] that the kind of caseinogen used in the basal diet for vitamin A testing is of the greatest importance in this respect. One sample of "vitamin-free casein," failed completely to fulfil the required conditions. Some of the rats grew well but the mean responses of groups of rats to different doses of cod-liver oil were slightly graded to the lower doses only and, at a comparatively low level, reached a limit which was far below any "normal" rate. The caseinogen was suspected of being the limiting factor and a fresh sample was used. "Light white casein" bought from the British Drug Houses Ltd. was extracted cold once with alcohol and four times with ether (24 hours each extraction), and the whole experiment repeated with satisfactory results. On the higher doses of cod-liver oil, the growth response of the groups might be considered almost "normal." It was probably as great as could ever be obtained immediately after the cessation of growth due to a deficiency of vitamin A. Moreover, the differences in response of different groups of rats to doses differing by 100 % were great enough to be considered significant even when the possible error of the estimation was allowed at 2ϵ where $\epsilon = \sqrt{\frac{\sum d^2}{n(n-1)}}$. This held through a range of doses from 0.25 mg. to 7.5 mg.

The extraction of the caseinogen was, however, both costly and laborious and it occurred to us that the greater growth-promoting power of "light white casein" formerly ascribed to the presence in it of vitamin A might, in

reality, be due to whatever constituted the difference between it and the "vitamin-free casein" which we have described in recent papers. We accordingly made comparisons in different ways between "light white casein" extracted as we were in the habit of extracting it and "light white casein" not treated in any way. We also compared untreated "light white casein" with some of the same batch heated in exactly the same way as Messrs Glaxo heat their caseinogen to make the "vitamin-free casein." We are satisfied that the extraction of the "light-white casein" is entirely unnecessary. It follows that the heating process of Messrs Glaxo is therefore unnecessary and moreover we have shown that it is slightly but definitely harmful to the caseinogen.

METHOD.

1. *Exp. 1. Comparison of caseinogens as the protein of a vitamin A-free diet.*

A large batch (2 cwts.) of "light white casein" (B.D.H.) was thoroughly mixed. A part of it was left untreated, a second part of it was extracted once with alcohol, 4 times with ether, cold (24 hours each extraction) and a third part was, through the courtesy of Messrs Glaxo, heated in thin layers as they heat their own caseinogen to render it "vitamin-free." The three parts are here referred to as

- (a) untreated "light white casein";
- (b) extracted "light white casein";
- (c) treated "light white casein."

The three forms of caseinogen were incorporated in vitamin A-free diets in the usual way (*i.e.* 15 % caseinogen, 73 % dextrinised rice starch, 8 % dried yeast and 4 % (Steenbock's 40) salt mixture; the diet was given to the rats *ad lib.* together with about 2 units vitamin D per rat twice a week). The three diets were tested on large groups of animals to determine (a) the average length of time required for the animals to cease growing, (b) the average weight at which the animals ceased growing. Table I gives a summary of the results of this experiment. Details are given in Tables II, III and IV. It is evident that (a) the times taken for groups of animals to cease growing and (b) the average weights at which these animals cease growing are the same

Table I. *Summary of results of Exp. 1.*

To compare the growth of groups of rats given a vitamin A-free diet containing

- (a) untreated "light white casein" (B.D.H.),
- (b) extracted "light white casein" (B.D.H.) (once alcohol, 4 times ether),
- (c) treated "light white casein" (B.D.H.) (heated in thin layers).

		Average of highest weights attained	Average weights at which considered steady	Average of times taken for weights to become steady
		g.	g.	(days)
(a) Untreated L.W.C.	±0.3	110.0	102.8	41.9
		105.5	99.7	53.8
(b) Extracted L.W.C.	±0.3	110.7	102.8	38.9
		101.6	96.1	43.8
(c) Treated L.W.C.	±0.3	82.1	77.1	34.2
		81.8	77.1	38.5

whether untreated or extracted "light white casein" is used in the basal diet. On the other hand, the animals whose basal diet contained the treated caseinogen ceased growing in a shorter time and at a lower weight than either of the other two groups. Two interpretations of this are possible. Either the "light white

Table II.

To show: (a) the average time taken by a group of rats to become steady in weight on a vitamin A-free diet containing untreated "light white casein"
 (b) the average weight at which these rats became steady.
 (c) the effect of giving 5 of these rats a daily dose of 0.1 g. of a vitamin margarine for 5 weeks.

"Light white casein," untreated in the vitamin A-free diet.

	Rat	Initial weight	Highest weight attained	Weight when considered steady	Time taken to become steady (days)	Dose	Response in 35 days
		g.	g.	g.			g.
♂	M 4804	50	133	132	45	—	—
♂	M 4853	43	139	136	59	—	—
♂	M 4852	49	139	128	50	—	—
♂	M 4898	45	132	109	49	—	—
♂	M 4900	47	125	114	42	—	—
♂	M 4856	46	113	112	45	—	—
♂	M 4855	47	137	117	48	—	—
♂	M 4899	41	93	91	45	—	—
♂	L 2	48	170	162	78	—	—
♂	2323	38	105	98	36	—	—
♂	M 4896	47	109	97	37	—	—
♂	M 4854	42	72	70	24	0.1 g. G. Ch. marg.	43
♂	M 4810	44	82	72	24	0.1 g. G. Ch. marg.	48
♂	2342	46	54	52	22	0.1 g. G. Ch. marg.	69
♂	M 4809	42	109	105	42	—	—
♂	M 4806	46	85	84	42	—	—
♂	M 4897	46	163	155	63	—	—
♂	M 4851	44	53	46	20	0.1 g. G. Ch. marg.	- 3, dead* in 2 days
♂	M 4811	46	77	73	24	0.1 g. G. Ch. marg.	42
♂	M 4895	(56)	(82)	(68*)	(42)	—	—
Av. = 45.1 Av. = 110.0 Av. = 102.8 Av. = 41.9							
♀	M 4864	49	110	107	56	—	—
♀	M 4803	44	109	90	66	—	—
♀	M 4865	44	124	120	67	—	—
♀	L 5	44	120	115	77	—	—
♀	M 4868	45	101	97	46	—	—
♀	M 4867	(47)	(125)	Discarded	(76)	—	—
♀	M 4804	41	111	103	42	—	—
♀	M 4802	42	105	104	50	—	—
♀	M 4807	42	93	91	42	—	—
♀	M 4808	45	85	80	42	0.1 g. G. Ch. marg.	16
♀	M 4805	40	97	90	50	—	—
♀	M 4866	(44)	—	(38*)	(18)	—	—
♀	L 6	(36)	—	(36*)	(13)	—	—
Av. = 43.6 Av. = 105.5 Av. = 99.7 Av. = 53.8 Av. = 43.6 (omitting*)							

* Rat died, not included in averages.

casein" contains vitamin A which has not been removed by extraction but has been destroyed by heat, or the "light white casein" does not contain any vitamin A and the heat has damaged it as protein or some unknown factor in it. The later experiments throw light on this matter.

2. *Exp. 2. The result of substituting untreated "light white casein" for (a) extracted "light white casein" or (b) treated "light white casein" in the vitamin A-free diets of rats which have ceased to grow on these diets.*

(a) Fifteen rats (6 bucks and 9 does) which had become steady in weight on a vitamin A-free diet containing extracted "light white casein" were given a diet containing untreated "light white casein," to see whether the amount

Table III.

To show: (a) the average time taken by a group of rats to become steady in weight on a vitamin A-free diet containing extracted "light white casein";
(b) the average weight at which these rats became steady;
(c) the effect of substituting untreated "light white casein" for extracted "light white casein" in this diet.

"Light white casein" in a vitamin A-free diet.							
Extracted					Untreated		
Rat	Initial weight g.	Highest weight attained g.	Weight when considered steady g.	Time taken to become steady (days)	Increase if any on this "casein" g.	Total response g.	Time taken for this response (days)
♂ M 4876	52	94	85	41	1	- 9	13
♂ M 4904	50	129	117	38	1	- 16	12
♂ M 4873	51	111	108	40	—	- 20	16
♂ M 4875	47	112	103	40	—	- 36	13
♂ M 4906	55	126	122	38	—	- 10*	18
♂ 2325	38	92	82	36	—	- 10*	2
♂ M 4902	(46)	(126)	(104*)	(35)	—	—	—
♂ M 4903	(50)	(101)	(84*)	(24)	—	—	—
♂ M 4874	(43)	(43)	(32*)	(28)	—	—	—
♂ M 4905	(49)	(49)	(39)	(15)	Died within 6 days of being put on a test; therefore discarded Found wandering in rat room; therefore discarded		
♂ 2344	(43)	(44)	(40)	(18)			
♂ M 4901	(40)	(40)	(34*)	(14)	—	—	—
Av. = 48.8 Av. = 110.7 Av. = 102.8 Av. = 38.9							
♀ M 4862	48	128	125	43	5	- 11	30
♀ M 4870	46	91	89	46	14	13	24
♀ M 4871	51	85	82	40	—	- 8	21
♀ M 4861	52	125	117	38	—	- 17	21
♀ M 4863	42	94	82	49	3	- 10	14
♀ M 4860	45	90	84	43	—	- 18	9
♀ M 4872	49	85	81	46	—	- 26*	7
♀ M 4869	50	88	82	40	—	- 22*	15
♀ M 4858	45	128	123	49	7	- 13	31
♀ M 4857	(47)	(47)	(36*)	(17)	—	—	—
Av. = 47.5 Av. = 101.6 Av. = 96.1 Av. = 43.8							

* Rat died, not included in averages.

of vitamin which might be present in the untreated sample would be sufficient to make the rats resume growth. The result has been summarised in Table III. It may be seen that 4 rats have increased in weight (5, 14, 3, 7 g. respectively), 2 have gained 1 g. which is not considered significant, 9 have lost weight. It is evident that the amount of vitamin A, if any, in untreated "light white casein" is negligible.

(b) Nineteen rats (7 bucks and 12 does) which had become steady in

weight on a vitamin A-free diet containing treated "light white casein" were given a diet containing untreated "light white casein." The response to this change was greater than the response when extracted was changed to untreated "light white casein." Seven rats increased in weight (11, 9, 14, 19, 16, 2, 5 g. respectively) and the rest lost weight or gained only 1 g.

Table IV.

To show (a) the average time taken by a group of rats to become steady in weight on a vitamin A-free diet containing treated "light white casein";
 (b) the average weight at which these rats became steady;
 (c) the effect of substituting untreated for treated "light white casein" in the diet;
 (d) the effect of giving 5 of these rats a daily dose of 0.1 g. of a vitamin margarine for 5 weeks.

Rat	Treated				Untreated			
	Initial weight g.	Highest weight attained g.	Weight when considered steady g.	Time taken to become steady (days)	Increase if any on this "casein" g.	Total response g.	Time taken for this response (days)	Response in 35 days g.
♂ M 4879	44	85	82	34	11	+ 10	16	—
♂ M 4800	44	100	95	43	—	- 17	16	—
♂ M 4798	47	92	79	34	1	- 7	25	—
♂ M 4878	51	60	57	32	—	- 5	17	—
♂ L 3	39	80	75	50	9	+ 5	16	—
♂ M 2324	38	64	64	36	—	- 12*	8	—
♂ M 4797	49	100	97	42	—	- 30*	18	—
♂ L 1	49	61	56	24	—	—	—	—
♂ M 4801	43	90	80	28	—	—	—	0.1 g. G. Ch. marg. 27
♂ M 4799	49	100	96	29	—	—	—	0.1 g. G. Ch. marg. 9
♂ M 4796	48	71	67	24	—	—	—	0.1 g. G. Ch. marg. 24
♂ M 4880	(53)	(112)	(102*)	(32)	—	—	—	0.1 g. G. Ch. marg. 35
♂ M 4877	(50)	(111)	(105*)	(32)	—	—	—	—
<hr/>								
	Av. = 45.5	Av. = 82.1	Av. = 77.1	Av. = 31.2				
♀ M 4888	44	71	69	31	14	+ 1	21	—
♀ M 4850	51	111	106	36	—	- 5	16	—
♀ M 4846	44	94	88	43	—	- 9	27	—
♀ M 4891	50	93	86	43	19	- 1	23	—
♀ M 4849	52	112	110	45	16	+ 5	28	—
♀ M 4848	49	85	82	43	—	- 10	13	—
♀ L 4	37	64	62	45	—	- 12*	11	—
♀ M 4890	42	59	50	24	—	- 5*	3	—
♀ M 2343	44	68	67	39	2	- 12*	12	—
♀ M 4887	45	77	74	31	—	- 2*	14	—
♀ M 4845	47	70	76	38	5	- 11*	11	—
♀ M 4844	48	48	45	17	—	—	—	—
♀ M 4892	47	102	95	66	—	- 10	3	0.1 g. G. Ch. marg. -1
♀ M 4893	(43)	(78)	(56*)	(24)	—	—	—	—
♀ M 4847	(49)	(102)	(86*)	(49)	—	—	—	—
♀ M 4889	(53)	(68)	(53*)	(24)	—	—	—	—
<hr/>								
	Av. = 40.2	Av. = 81.8	Av. = 77.1	Av. = 38.5				

* Rat died, not included in averages.

It is concluded that there is less difference between the extracted and untreated caseinogen than between the treated and untreated. Indeed the difference between the extracted and untreated is considered insignificant.

3. *Exp. 3. The effect of giving equal doses (0.1 g.) of a vitamin margarine to rats which had ceased to grow on a vitamin A-free diet containing (a) untreated "light white casein" and (b) treated "light white casein."*

Four bucks and one doe were used in each part of this experiment. (A fifth buck in one group died after 3 days and was therefore discarded.) The result is summarised in Table V. Details are given in Tables II and IV.

This result is open to the interpretation that the small amount of vitamin A which might be present in the untreated "light white casein," although not enough to prevent cessation of growth, might supplement the vitamin A of the vitamin margarine and thus cause the greater growth of this group of animals. This explanation, however, seems to be impossible in view of the work described in earlier papers, for we have found that even when excessive amounts of vitamin A in the form of 0.2 g. cod-liver oil daily have been given, we have obtained remarkable increases in growth rates on substituting "light white casein" (B.D.H.) for the "vitamin-free casein" (Glaxo). It seems much more probable that the treated caseinogen has been altered in some way so that it is less adequate for growth than the untreated form.

Table V.

Caseinogen	Dose of vitamin margarine	Average increase in weight of the group in 5 weeks
Untreated	g. 0.1	g. 43.6
Treated	0.1	18.8

4. *Exp. 4. The effect of giving cod-liver oil to rats which have become steady in weight on a vitamin A-free diet containing (a) untreated "light white casein" and (b) extracted "light white casein."*

Where the same dose of cod-liver oil has been used for comparing the two forms of caseinogen, results are, of course, directly comparable. Where different doses have been used, the results are compared by reference to a curve relating response to dose of oil. The curve which we have worked out for our own laboratory has already been described [Coward, Key, Dyer and Morgan, 1930]. The equation for this curve is $y = 12.1 + 40.24 \log x$, where y = the mean increase of a group of animals all given the same dose, and x = the dose of cod-liver oil in mg. It has been found that this curve can be resolved into two somewhat similar curves, one for the bucks used in its construction and one for the does. These curves are:

$$y = 11.3 + 50.3 \log x \text{ (bucks).}$$

and

$$y = 12.4 + 27.4 \log x \text{ (does).}$$

When it has not been possible to use equal numbers of bucks and does for a test, comparisons may be made by means of the two curves. The average of the results will then be more accurate than a direct comparison made through the original curve. We now express the vitamin A potency of any sample examined in our laboratory as a percentage of the potency of the oil which we used in the making of these curves and which we have temporarily adopted as a standard. We find the mean increase of a group of rats (about 10) given a certain dose of cod-liver oil daily for 5 weeks, then find by the curves what dose of our standard oil would have given that increase and thus determine the relative potency of the oils. In this way we found two oils, B and C, to have the same vitamin A potency relative to oil A whether they were tested on a diet containing untreated "light white casein" or on one containing extracted "light

white casein." Table VI gives a summary of these figures and Table VII gives the details.

This result is regarded as additional evidence that the amount of vitamin A in "light white casein," if any, is negligible, and that the laborious and expensive process of extracting "light white casein" is superfluous.

Table VI.

Potency expressed as a percentage of the potency of oil A when examined on basal diets containing

Oil	(a) Untreated caseinogen	(b) Extracted caseinogen
B	83.05	72.9
C	173.8	189.6

Table VII.

To show that the vitamin A potency of an oil relative to another oil is the same whether determined with untreated "light white casein" or with extracted "light white casein" in the basal diet.

Oil	Dose mg.	No. of bucks	Average increase in 5 weeks g.	Dose of oil A which brought about this increase mg.	No. of does	Average increase in 5 weeks g.	Dose of oil A which brought about this increase mg.	Relation to oil A (%) according to	
								Bucks	Does
B	1.0	10	4.2	0.723	9	11.8	0.95	72.3	95
	untreated caseinogen							Average = 83.65	
								Weighted mean = 83.05	
	4.0	4	35.75	3.06	5	24.6	2.79	76.5	69.7
	extracted caseinogen							Average = 73.1	
								Weighted mean = 72.9	
C	1.0	6	27.7	2.118	13	20.2	1.9275	211.8	192.75
	untreated caseinogen								
	5.0	7	54.9	7.357	11	36.2	7.393	147.1	147.9
	untreated caseinogen							Average = 174.9	
								Weighted mean = 173.8	
	5.0	13	61.4	9.91	4	37.25	8.075	198.2	161.5
	extracted caseinogen							Average = 179.85	
								Weighted mean = 189.6	

SUMMARY.

It has been shown in several ways that "light white casein" (B.D.H.) may be safely used without extraction for vitamin A tests.

1. Large groups of rats were given a vitamin A-free diet containing (a) extracted or (b) non-extracted "light white casein" (B.D.H.). They became steady in weight at about the same weight and in about the same length of time.

2. Rats which had become steady in weight on a vitamin A-free diet containing extracted "light white casein" were given an exactly similar diet

but containing non-extracted "light white casein"; they did not resume growth but continued to lose weight.

3. The estimation of the vitamin A potency of a sample of cod-liver oil relative to another sample used as a standard of reference in these laboratories was the same whether extracted or non-extracted "light white casein" was used in the basal diet.

The extraction of the caseinogen has not removed the growth-factor whose existence has recently been demonstrated by Coward *et al.* [1929].

The heating of "light white casein" by Messrs Glaxo in the way in which they prepare their "vitamin-free casein" only partially destroyed this factor.

REFERENCES.

- Coward, Key, Dyer and Morgan (1930). *Biochem. J.* **24**, 1952.
 ——— and Morgan (1929). *Biochem. J.* **23**, 695.
 Fisher (1925). *Statistical methods for research workers.*

APPENDIX.

The method of determining the equations of the curves.

The mean increases (y) of the groups of rats given different doses of cod-liver oil for a period of 5 weeks were plotted against the logarithms of the doses of oil given. They fell practically on a straight line. The best straight

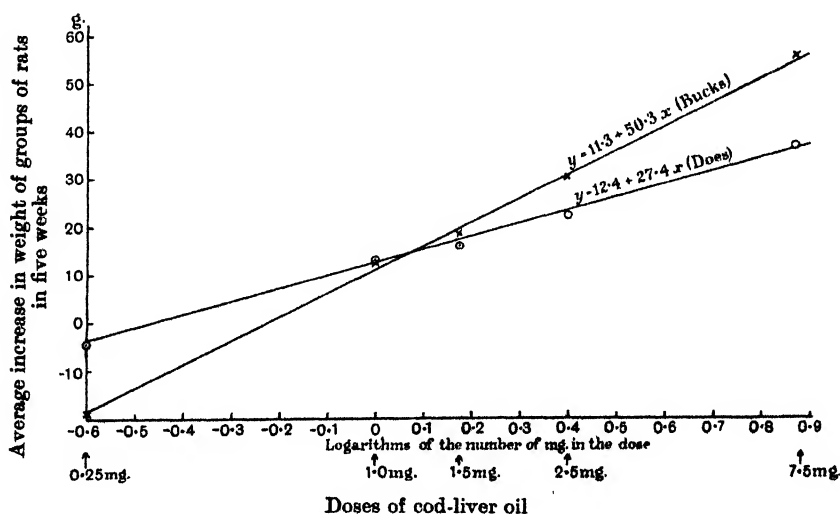


Fig. 1. Curves relating mean increases in weight in 5 weeks of groups of rats (bucks and does respectively) to the logarithms of the doses of cod-liver oil given.

The more nearly the mean increases in each curve lie in a straight line when plotted against the logarithm of the dose, the more truly is the response expressed by the corresponding logarithmic equation.

line through the five points was obtained by the method described by Fisher [1925, p. 117].

$$y = a + b(x - \bar{x})$$

where

$$a = \bar{y} = \text{mean value of } y$$

$$\bar{x} = \text{mean value of } x$$

and

$$b = \frac{\sum y(x - \bar{x})}{\sum (x - \bar{x})^2}.$$

This gave the equation $y = 12.1 + 40.24x$ (where x is the logarithm of the dose of oil), whence is derived the equation $y = 12.1 + 40.24 \log x$ to represent the relation between response and dose of oil.

This equation was obtained from results on groups of rats consisting of approximately equal numbers of bucks and does. It occurred to us that it might be useful to have separate equations for results from bucks and does respectively. We therefore plotted the mean increases of the bucks and does in each group separately against the logs of the doses. These also gave two practically straight lines (Fig. 1) which were determined by the same method:

$$y = 11.3 + 50.3x \text{ (bucks),}$$

and

$$y = 12.4 + 27.4x \text{ (does).}$$

The equations relating response to dose are therefore:

$$y = 11.3 + 50.3 \log x \text{ (bucks),}$$

and

$$y = 12.4 + 27.4 \log x \text{ (does).}$$

Two points of interest arise from a consideration of these curves. Firstly, only about 15 animals were used for each point on the curve. The smoothness of the curve would appear to indicate therefore that a very reliable average can be obtained from 15 animals, a comparatively small number for a biological estimation. Secondly, the two curves indicate that the bucks respond more vigorously than the does to the higher doses of vitamin A but it is remarkable that the does respond more vigorously than the bucks to the lower doses. This led us to wonder whether the mortality among the bucks had been greater than that among the does in work on vitamin A deficiency. We therefore counted the numbers of bucks and does respectively that had died during the test period (5 weeks) of the experiment and also those from similar experiments that we have just concluded. We found that, of 288 bucks which had been used for testing various doses of cod-liver oil, 57 or 19.8 % had died, while of 277 does used in the same experiments (in more or less equal numbers on each test) 37 or 13.4 % had died. This difference appears to be significant.

Dr J. Gaddum of the National Institute for Medical Research has made the following calculation from the data and has concluded that the difference is significant and, therefore, that male rats require more vitamin A to prevent death than do female rats.

Group of rats	Dose of cod-liver oil given daily mg.	No. of bucks in group	Total no. of bucks dying during test	% no. of bucks dying during test	No. of does in group	Total no. of does dying during test	% no. of does dying during test
1	1 oil B	12	2	16.7	10	1	10.0
2	4 „ B	5	1	20.0	5	0	0.0
3	0.5 „ C	11	4	36.4	14	2	14.3
4	1.0 „ C	9	3	33.3	14	1	7.1
5	5.0 „ C	14	1	7.1	4	0	0.0
6	5.0 „ C	7	0	0.0	12	1	8.3
7	0.5 „ D	9	1	11.1	13	1	7.7
8	1.0 „ D	10	2	20.0	13	2	15.4
9	0.5 „ E	13	3	23.1	16	0	0.0
10	1.0 „ E	13	3	23.1	13	2	15.4
11	0.1 „ F	17	7	41.2	11	0	0.0
12	0.5 „ F	9	1	11.1	13	1	7.7
13	1.0 „ F	16	2	12.5	6	0	0.0
14	1.0 „ G	11	3	27.3	11	3	27.3
15	1.0 „ H	10	0	0.0	12	3	25.0
16	1.0 „ J	11	1	9.1	12	0	0.0
17	1.0 „ K	2	0	0.0	9	1	11.1
18	0.25 „ A	18	11	61.1	13	6	46.2
19	1.0 „ A	23	6	26.1	14	7	50.0
20	1.5 „ A	13	3	23.1	22	5	22.7
21	2.5 „ A	21	2	9.5	11	0	0.0
22	7.5 „ A	19	1	5.3	12	0	0.0
23	20.0 „ A	15	0	0.0	17	1	5.9
		288	57		277	37	

If the sexes were equally sensitive, the number of groups in which the mortality of the males was larger than that of the females should equal the number of groups in which the mortality of the females was larger than that of the males. Actually, more males than females died in 17 groups, more females than males died in 5 other groups and in 1 group the numbers of males and females that died were equal. Thus:

Observed	More males dead	More females dead
Calculated on assumption that mortalities are equal							17.5 (a)	5.5 (b)
							A (c)	A (d)

where A is a very large number.

To find the probability that these two results are compatible [see Fisher, 1925, p. 84]:

$$\begin{aligned}\chi^2 &= \frac{(ad - bc)^2 (a + b + c + d)}{(a + b)(c + d)(a + c)(b + d)} \\ &= \frac{[A(17.5 - 5.5)]^2 (2A)}{23 \times 2A \times A \times A} \quad \text{(neglecting } a + b \text{ in comparison with the large number } A) \\ &= \frac{12^2}{23} = 6.26.\end{aligned}$$

The table of χ^2 shows (when $n = 1$) that the probability of this value of χ^2 being due to chance is between 1/50 and 1/100.

LXV. THE RELATION BETWEEN AMYLASE AND LACTIC ACID FORMATION IN MUSCLE.

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(Received February 28th, 1931.)

THE work reported in this paper was begun with the intention of investigating further the nature of the inhibition of lactic acid formation in muscle which is brought about by pancreatic and other extracts. This phenomenon had been shown by Case and McCullagh [1928] to be caused by the amylase present in such extracts, and it was demonstrated that similar inhibition could be brought about by amylase from various other sources, both animal and plant. In an earlier paper the mode of action of the "pancreatic factor," then unidentified with amylase, had been described by McCullagh [1928] as a suppression of the esterification of phosphoric acid; in other words, as a prevention of the formation of hexosephosphates rather than a prevention of their breakdown to lactic acid. This hypothesis was adhered to in the subsequent communication.

Harrison and Mellanby [1930] agreed that the substance responsible for the inhibition is amylase, but they did not regard as admissible the above interpretation of the manner in which it acts. Actually it appears that their criticism was based, in large part, upon a misunderstanding with regard to the meaning which was intended to be borne by the explanation in question.

It is clear that a statement such as that "inhibition of lactic acid formation depends upon the suppression of esterification of carbohydrate and phosphoric acid" can be presumed to apply only to those cases where inhibition does in fact occur; that is to say, to cases where the carbohydrate involved is starch or glycogen. When activated glucose is the substrate, amylase causes no inhibition, as was shown both by Case and McCullagh and by Harrison and Mellanby. From these circumstances, taking into consideration the fact that starch and glycogen are natural substrates for amylase, while glucose and other hexoses are not, it seems a reasonable deduction that the inhibition observed is related to an interaction of some kind between polysaccharide and amylase, in virtue of which the lactic acid-forming process is checked at its first stage, namely that of esterification. In a sense, it may be said that Harrison and Mellanby are agreeing to this when they put forward the suggestion that "an explanation of the experimental observations can be based

on the well-known fact that amylase breaks down starch to maltose," which substance as Meyerhof has shown is practically unacted upon by the muscle enzyme system. This explanation, which as Harrison and Mellanby point out seems obvious enough at first sight, is in the present writer's opinion inadequate, in such a simple form, to account for the complete and instantaneous inhibition of lactic fermentation which is produced by very small amounts of added amylase. It is not conceivable that immediately after the addition to the system of amylase all the starch is at once transformed to maltose and in that way removed from the sphere of action of the esterifying and other enzymes. It seems much more likely that the explanation is to be sought in the formation of some amylase-starch compound or complex, whereby the starch is protected from and rendered inaccessible to the muscle enzymes. That something of this nature indeed takes place is suggested by the fact that even in the presence of considerably more amylase than is necessary to produce complete inhibition of esterification and lactic acid formation, the characteristic blue compound with iodine can be obtained, thus demonstrating the continued existence in the solution of unhydrolysed starch. The breakdown of the starch into maltose proceeds slowly and can be followed by the iodine method or a reduction method, while the lactic acid formation is completely checked *ab initio*.

The experimental work to be described was originally commenced in order to produce fresh justification for this point of view, but as will be seen, results were obtained which throw light from a different angle on the problem of the relation between amylase and lactic acid formation in muscle.

EXPERIMENTAL.

In order to test the theory of Harrison and Mellanby referred to above, namely that maltose formation is responsible for the inhibition, the following experiment was designed. If one were to incubate starch for a time with amylase in sufficient quantity to cause complete inhibition of lactic acid formation by a given muscle extract, and then, after this preliminary incubation, remove or destroy the amylase, it should be possible to show that there still remains starch which can now yield lactic acid when incubated with muscle extract.

Table I sets forth the results of one experiment carried out on these lines.

Table I.

					mg. lactic acid in 15 cc. sample		
					Before incubation	After incubation	Change
1.	Muscle extract + starch + buffer + water				10.9	27.8	16.9
2.	" " + 5 cc. amylase				11.1	12.3	1.2
3.	" (starch + buffer + 5 cc. amylase)	A			11.2	28.8	17.6
4.	" " "	B			10.9	23.3	12.4
5.	" " "	C			10.9	18.2	7.3
6.	" " "	D			11.2	14.6	3.4
7.	" " "	E			11.3	13.9	2.6

In preparation A the starch, buffer and amylase were mixed and incubated together at 27° for 1 minute, then boiled for 5 minutes in order to destroy the amylase. B, C, D and E received similar treatment, the incubation times being 5, 10, 20 and 30 minutes respectively before the 5 minutes' boiling. All were cooled to room temperature before the muscle extract was added. The composition of the final mixture was in all cases:

25 cc. muscle extract,
10 cc. 2 % starch,
10 cc. phosphate buffer.
5 cc. water or amylase.

Incubation was carried out for half an hour at 27°. The technique of sampling and of estimation of lactic acid was exactly as described in former work [Case, 1929], as was the preparation of the muscle extract and the composition of the buffer. This applies to all the lactic acid experiments reported in this paper.

The amylase employed was a diluted preparation of saliva, 5 cc. of which had been shown by a preliminary test to give 100 % inhibition of lactic acid formation with an average muscle extract.

It follows from these results that for a period of at least half an hour after the addition of amylase, starch remains present in the system and can be utilised by the lactic acid-forming enzymes if the amylase is removed. It would seem then that the starch is in some way held by the amylase prior to any destruction, and held in such a way that it is inaccessible to the myozymase system. It was a conception of this kind that was foreshadowed in the concluding sentence of Case and McCullagh's [1928] paper, *viz.* "it may be that some molecular union between amylase and its substrate, prior to enzymic hydrolysis, can be demonstrated."

All that has been said above applies with equal force to the case of glycogen, which of course is the naturally-occurring substrate for the muscle enzymes. An experiment similar in every respect to that of which Table I is the protocol, except that starch was replaced by glycogen, yielded substantially identical results.

It is well known that the tissue of numerous organs, including muscle, contains amylase in varying quantities. With regard to muscle, it becomes of interest to inquire as to the nature of the rôle played by its amylase.

Ronzoni [1929] believes that "hydrolysis of starch is a necessary step in glycolysis of muscle extract." In support of this view, she claims to have produced an acceleration of esterification by adding amylase to muscle extracts which, owing to short periods of extraction, were deficient in this enzyme. The present writer has, however, never succeeded in obtaining confirmation of this. It is interesting to note in this connection that preparations of amylase from muscle itself, made by glycerol extraction or other means, resemble amylases from other sources in that their addition in sufficient quantity to

the myozymase system produces inhibition of lactic acid formation. This fact was realised by Ronzoni and is mentioned in her paper.

Now the muscle enzyme system as a whole is strikingly thermolabile. Heating for a few minutes at 37° is sufficient to destroy its power of forming lactic acid from carbohydrate, or of esterifying phosphate. Muscle-amylase itself has a much greater degree of stability. It should be possible therefore, by heating muscle extract for, say, a quarter of an hour at 37°, to leave the amylase activity intact while destroying the lactic acid-forming property of the extract. A comparison of the rates of starch or glycogen destruction in an extract so treated, and at the same time in an unheated portion of the same extract, should then give significant results. If in both cases carbohydrate disappeared at the same rate, the fact would be an indication that amylase action is the first step in the degradation of starch. In order to render such a conclusion admissible, it would be necessary to ensure that the starch concentration was small enough to be below the saturation value for the amount of enzyme present.

In Table II are embodied the results of a number of experiments made to test this point. In each instance a part of the muscle extract was heated for 15 minutes at 37°, the remainder being kept at 0°. After cooling the first portion to 0° again, incubation mixtures were set up consisting of 20 cc. of muscle extract, 10 cc. of phosphate buffer and 10 cc. of 0.4 % starch. The final concentration of starch was thus 0.1 %. The incubations were carried out at 27°, and at intervals 0.5 cc. samples were tested with iodine. In each case the time was noted at which the addition of iodine produced no coloration.

Table II.

		Time (in mins.) for achromic point with iodine				Time (in mins.) for achromic point with iodine	
		Normal extract	Heated extract			Normal extract	Heated extract
A	p_H			C	p_H		
	6.0	41	44		6.0	35	35
	7.0	37	38		7.0	36	39
	8.0	32	32		8.0	30	33
B	6.0	20	21	D	6.0	13	12
	7.0	20	18		7.0	13	14
	8.0	18	20		8.0	12	13

A, B, C and D represent different muscle extracts made at different times.

Variations of p_H between the above limits, although in each case altering to some extent the actual times taken for the attainment of the achromic point, are seen to make no difference to the relative values for heated and unheated extracts.

The important point emerging from these figures is that in every instance the rates of disappearance of starch are the same, in spite of the fact that in one case lactic acid is being produced in the ordinary way, while in the other case nothing is occurring beyond amylolytic hydrolysis of the starch. This

seems to furnish strong evidence in support of the conclusion that the breakdown of starch to lactic acid depends primarily upon the amylase present in the extract.

Table III demonstrates that lactic acid production is, in fact, eliminated in the heated extracts. The figures given are for incubation mixtures A to D of the previous table, the p_H being 7.0.

Table III.

	Lactic acid production in 1 hour (mg. in 15 cc. sample)	
	Normal extract	Heated extract
A	4.9	0.5
B	7.0	0.8
C	5.5	0.5
D	8.7	1.0

The figures here given for "heated extract" are not quantitatively reliable, on account of the fact that the amounts of lactic acid are too small to be estimated accurately. The significance of the table, however, is not thereby affected.

A somewhat similar series of experiments was next performed, with the difference that instead of starch, glycogen was employed, in a concentration of 0.1 %. As before, normal and heated extracts were incubated with buffer (p_H 7.0) and glycogen. Instead of following glycogen disappearance by an iodine method, 5 cc. samples were taken initially and at periods of 5, 10 and 30 minutes. The glycogen in these was estimated by the modification of Pfüger's [1909] method described by Barbour [1929]. The samples were delivered directly into 5 cc. of hot potassium hydroxide solution, thus preventing any further enzymic activity.

The results of these experiments are given in Table IV. As in Table II, E, F and G are separate muscle extracts.

Table IV.

Period of incubation in minutes	% glycogen in solutions							
	Normal extract				Heated extract			
	0	5	10	30	0	5	10	30
E	0.094	0.077	0.061	0.005	0.095	0.079	0.060	0.004
F	0.096	0.081	0.069	0.024	0.096	0.080	0.070	0.030
G	0.096	0.070	0.048	0.000	0.097	0.068	0.050	0.000

Here again we find a parallel disappearance of glycogen in the corresponding cases where the muscle enzyme has and has not been heated for 15 minutes at 37°; these results therefore corroborate those of Table II.

If the conclusion just drawn is correct, it is to be expected that any agency which suppresses the amylolytic activity of a muscle extract would suppress in corresponding degree the formation of lactic acid. Actually to remove the

amylase without affecting the other components of the system would be a difficult task; fortunately, however, another way out of the difficulty is open.

It has been known for some time that muscle-amylase, in common with liver-amylase, differs in some respects from the enzyme obtained from pancreas, saliva, malt and other sources. Barbour [1929] made a study of glycogenolysis by muscle-amylase, and found that the sole product is a trisaccharide, which seems not to be identical with any sugar hitherto described; though Lohmann [1926] had reported among the products of glycogenolysis in muscle extracts a trisaccharide which may have been the same as that described by Barbour. Lohmann at the time suggested that the substance might be identical with the amylotriase of Pringsheim, but Barbour's more complete investigation of its properties appears to render this unlikely. If glycogen is hydrolysed with pancreatic or salivary amylase the products consist mainly of isomaltose with a little glucose; no trisaccharide is formed.

It was found possible to confirm Barbour's work, and by starting with several grams of glycogen to obtain relatively large amounts of a substance answering to his description of the trisaccharide. In general, glycerol extracts of finely minced rabbit muscle were employed and were incubated for 24 hours or longer with phosphate buffer (p_H 6.5) and 5 % glycogen. The trisaccharide was isolated from the digest according to the directions given by Barbour.

Now Wohl and Glimm [1910] showed that in the case of malt-amylase, the addition of maltose, the chief product of hydrolysis, caused inhibition of the amylolytic activity when starch was the substrate; this fact was made use of by Case and McCullagh [1928]. In a similar way, addition to muscle-amylase of the trisaccharide referred to above should depress the activity of the amylase. That this is so was shown by Barbour, and the following figures obtained during the course of the present work afford confirmation.

Table V.

Incubation period (hours)	Glycogen content (%)			Hydrolysis (%)	
	0	5	20	5	20
Control	0.98	0.64	0.15	35	85
Trisaccharide	0.97	0.95	0.97	2	0
Glucose	0.97	0.66	0.20	32	79
Maltose	0.98	0.66	0.23	33	77
Sucrose	0.96	0.68	0.22	29	77

In this experiment each flask contained 5 cc. of muscle-amylase (glycerol preparation) and 5 cc. of 2 % glycogen (made up in phosphate buffer of p_H 7.0). The different sugars (0.5 g. of each) were dissolved in the glycogen solutions before mixing with the enzyme. The mixtures were incubated at 27° and the glycogen was estimated in 3 cc. samples by the method referred to earlier in the work.

It is clear that the trisaccharide exerts a marked specific inhibitory action upon muscle-glycogenase.

The next step was to try the effect of adding trisaccharide to incubation

mixtures of glycogen and myozymase. To this end tubes were set up as follows (S represents trisaccharide):

1.	10 cc. muscle extract + 5 cc. 2 % glycogen + 5 cc. buffer	
2.	"	+ 0.25 g. S
3.	"	+ 0.5 g. S
4.	"	+ 1.0 g. S
5.	"	+ 1.0 g. glucose
6.	"	+ 1.0 g. maltose
7.	"	+ 1.0 g. sucrose
8.	" + 5 cc. water	+ 0.25 g. S
9.	"	+ 0.5 g. S

The production of lactic acid during one hour at 27° is shown in Table VI.

Table VI.

Tube No.	mg. lactic acid in 10 cc. sample		
	Before incubation	After incubation	Change
1	6.5	19.4	12.9
2	6.4	16.8	10.4
3	6.7	7.4	0.7
4	6.5	7.1	0.6
5	6.5	17.2	10.7
6	6.2	16.9	10.7
7	6.4	18.5	12.1
8	6.8	6.9	0.1
9	6.4	6.9	0.5

Here again it is seen that the addition of trisaccharide in a concentration of 2.5 %, has completely suppressed the formation of lactic acid from glycogen, while the other sugars tried have had no such effect. Tubes 8 and 9 are intended to demonstrate that the trisaccharide itself is not acted upon by muscle enzymes.

This experiment was repeated using half quantities throughout, and with the addition of sodium fluoride in 0.02 *M* concentration. Instead of lactic acid, phosphates were determined before and after the incubation by the Briggs-Bell-Doisy colorimetric method. The results, as is apparent from the next table, bear out those of Table VI; that is to say, esterification has been inhibited in the systems containing the trisaccharide, but in no others.

Table VII.

Tube No.	mg. P as free phosphate in 3 cc. sample		
	Before incubation	After incubation	Change
1	1.02	0.40	- 0.62
2	1.04	0.52	- 0.52
3	1.03	1.00	- 0.03
4	1.03	1.02	- 0.01
5	1.04	0.55	- 0.49
6	1.02	0.57	- 0.45
7	1.01	0.54	- 0.47
8	1.05	1.02	- 0.03
9	1.05	1.03	- 0.02

DISCUSSION.

Considering jointly the significance of the experimental results embodied in Tables V, VI and VII, one is led to the conclusion that since lactic acid production in muscle is inhibited by the trisaccharide, and the trisaccharide inhibits the activity of muscle-glycogenase, of whose action upon glycogen it is the end-product, then the first stage in the breakdown of glycogen into lactic acid is one dependent upon amylolytic activity. This is the same conclusion that was arrived at from a consideration of the facts reported in Tables II, III and IV, and up to a point is in agreement with Ronzoni's view of the situation. But whereas Ronzoni postulates amylolytic hydrolysis as a necessary preliminary, the present writer is not of this opinion. As has been said, it seems fairly certain from Barbour's work that the only product of glycogenolysis by muscle-amylase is the trisaccharide; it appears that the intermediate production of dextrans does not occur. The trisaccharide itself is incapable of being further acted upon by the muscle enzymes, as is shown in Tables VI and VII. It may be said in parenthesis that Lohmann's trisaccharide was also non-fermentable.

The position may then be summarised by the statement that on the one hand the action of muscle-amylase is a necessary preliminary to the formation of lactic acid from glycogen, while on the other hand the product of the hydrolysis of glycogen by muscle-amylase is one which cannot be attacked by myozymase. The inference is therefore that this product, the trisaccharide, is not formed in the process of lactic acid production. Since it is the only product of glycogenolysis by muscle-amylase, it follows that in all probability hydrolysis does not occur at all. The muscle-amylase, if this is the case, is not functioning in the manner that normally would be expected of an amylase.

The suggestion put forward is that we have in muscle a system of enzymes working not only in a coordinated consecutive manner but in a conjoint and simultaneous way, in the sense that the amylase and another component of the system, presumably the esterifying enzyme, are so closely correlated and interdependent in their activity that they may, in fact, be considered to form a single colloid complex or aggregate. It is a generally accepted hypothesis that an enzyme molecule proper is attached to an inactive associated structure, probably of protein. It is necessary to imagine then that in this particular case both the amylase and the esterifying enzyme are attached to the same colloid structure and constitute to that extent a single entity. The first step in the transformation of glycogen to lactic acid seems to be then not merely a union of the carbohydrate with amylase, but a simultaneous union with both amylase and another enzyme component of myozymase—presumably involving two different sites on the carbohydrate molecule. In this connection it is tempting to allude to the extreme thermolability of the myozymase system as a whole. The very pronounced effect of even short exposure to quite low degrees of heat (*e.g.* 37°) is highly unusual, even in enzyme chemistry,

and it is difficult to regard this effect as consistent with the destruction of some enzyme component; it seems a more likely hypothesis that the exaggerated deleterious effect of temperature occurs as the result of the rupture of some delicate colloidal complex between one part of the system and another.

SUMMARY.

1. The nature of the inhibition of lactic acid formation in muscle caused by amylase is further investigated and the conclusion is drawn that an enzyme-substrate compound is formed prior to the hydrolysis of starch or glycogen by amylase.

2. Evidence is adduced in support of the hypothesis that the amylase of muscle is concerned in the process whereby lactic acid is produced from carbohydrate.

3. The concept of two enzymes functioning in a mutually dependent manner and not as individuals is introduced and is briefly discussed with reference to the case of muscle.

I am indebted to Sir F. G. Hopkins for his interest and encouragement, and to Prof. J. B. S. Haldane for much stimulating advice.

REFERENCES.

- Barbour (1929). *J. Biol. Chem.* **85**, 29.
Case (1929). *Biochem. J.* **23**, 210.
—— and McCullagh (1928). *Biochem. J.* **22**, 1060.
Harrison and Mellanby (1930). *Biochem. J.* **24**, 141.
Lohmann (1926). *Biochem. Z.* **178**, 444.
McCullagh (1928). *Biochem. J.* **22**, 402.
Pflüger (1909). *Pflüger's Arch.* **129**, 362.
Ronzoni (1929). *Proc. Soc. Exp. Biol. Med.* **26**, 356.
Wohl and Glimm (1910). *Biochem. Z.* **27**, 349.

LXVI. THE EFFECT OF STARVATION, PHLORIDZIN, THYROID, ADRENALINE, INSULIN AND PITUITRIN ON THE DISTRIBUTION OF GLYCOGEN IN THE RAT.

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THE effect of starvation, phloridzin and diabetes on the main glycogen stores in the liver and skeletal muscles was early established and latterly the effects of insulin and adrenaline on these organs have been intensively studied. A few investigations have been concerned with other organs, such as the heart in diabetes, but both early and recent work has been confined mainly to the liver and skeletal muscles or to the total glycogen content of the body. Thus, although glycogen has been demonstrated in every tissue, we can find no record of an attempt to follow its simultaneous changes in a number of representative organs, and we have made this the subject of the present investigation. Besides the liver and skeletal muscles, therefore, we have studied the heart, the stomach (as representing involuntary muscle), the spleen and the kidney, and a few observations have also been made on the brain, uterus and testicle. The uterus, containing as it does less non-muscular tissue than the stomach, might have been a better representative of involuntary muscle, but for routine purposes it is too small and its use would have limited us to female rats. The kidney and spleen were selected as non-muscular organs with no specialised "glycogen function" such as the liver possesses.

Our first intention was to investigate the glycogen changes in these organs produced by insulin and adrenaline, but a survey of the literature showed that such a comprehensive study had never been undertaken in starvation, or under the action of phloridzin or thyroid and we turned therefore to these first of all.

TECHNIQUE.

(1) *Animals.* Standard young albino rats (80–150 g.) obtained from the Glaxo laboratories were used throughout. The majority were males. They were fed on an excess of white bread soaked in milk and they increased in weight satisfactorily on this diet. The "fully fed" rats of our experiments have been killed 4 hours after receiving their food in the morning. At this time their

stomachs always contained much partially digested bread. The "24 hours' starved" and "48 hours' starved" animals were similarly fed in the morning, placed in a separate cage with water alone at room temperature 4 hours later, and killed after a further 24 or 48 hours.

(2) *Methods.* The rats were stunned by a blow on the head and immediately decapitated, and the sugar was determined in the blood from the carotid artery. Samples of the representative organs were rapidly removed and plunged into tared tubes containing 1 cc. of boiling 60 % potassium hydroxide. If the stomach contained food it was rapidly washed and dried. The time between death and the immersion of the last sample varied between 40 and 60 seconds, except in the case of the uterus. It took nearly 2 minutes to dissect out this organ cleanly.

We are aware now that this technique shows the glycogen in the muscle to be lower than it really was during life owing to the convulsive struggles of the stunned animal. Our results are however comparable with each other and show clearly the gross changes brought about by starvation *etc.* We do not lay great stress on small differences in the skeletal muscles until they have been confirmed by one or other of the improved techniques now available.

After 3-5 minutes in the boiling potassium hydroxide the tissues were completely dissolved. The tubes were then cooled and weighed, the weight of the sample being obtained by difference. The weights of liver and kidney taken were approx. 1 g.; of the stomach, heart, and skeletal muscles (adductor group of right thigh) 0.5 g. After weighing the tubes were again put into a water-bath at 100° for 3 hours when they were removed, and alcohol was added at once till the final concentration was 80 % (see below). After standing overnight the tubes were centrifuged, and the precipitate was washed twice with 5 cc. of 80 % alcohol *plus* 1 drop of saturated sodium chloride. Dilute hydrochloric acid was then added, and the glycogen was hydrolysed for 3 hours in the usual manner: the liquid was neutralised, made up to a definite volume and the glucose estimated by MacLean's method. It will be seen that the usual micro-Pflüger technique has been followed except in the use of 80 % instead of 66 % alcohol to precipitate the glycogen. This has been shown by Holmes [1929] to give a much more complete precipitation, and when working with very small amounts of glycogen this is a matter of great importance. Table I shows the results of precipitating and washing portions of the same liver (a) with 66 % alcohol; (b) with 80 % alcohol.

Table I. *Effect of concentration of alcohol on precipitation of glycogen.*

Glycogen % (liver) of rats starved 24 hours													Average
80 % alcohol	0.25	0.26	0.20	0.21	0.20	0.20	0.24	0.30	0.26	0.30	0.25	0.24	
66 % "	0.17	0.16	0.12	0.21	0.11	0.09	0.16	0.12	0.12	0.14	0.13	0.14	
Animals fully fed													
80 % alcohol	3.17	2.19	3.08	4.95	4.35	3.56							3.55
66 % "	2.86	2.68	2.79	5.34	3.65	3.52							3.47

In livers with a low glycogen content, the weaker alcohol gives much lower figures, but this difference is not seen when considerable amounts of glycogen are present. The use of the stronger alcohol, therefore, seems important only to remove the last traces of glycogen.

EXPERIMENTS AND RESULTS.

(1) *The Control Rats.* (a) fully fed; (b) 24 hours' starved; (c) 48 hours' starved; Table II A.

Although the "standard" rat has often been used for glycogen investigations it has been necessary to establish our own controls especially in the case of the heart, stomach and kidney. The results are given in Table II A. These controls do not apply to the experiments with adrenaline, pituitrin and insulin which were done on larger rats and at another time of year. Further controls for these experiments were performed and the results are tabulated beside the corresponding experiments.

Table II.

A. Controls.

Mean % of glycogen and average deviations from the mean.

Rats, treatment and number	Liver	Muscle	Heart	Stomach	Kidney
Fully fed (12)	2.3 (± 0.7)	0.54 (± 0.1)	0.52 (± 0.17)	0.21 (± 0.04)	0.18 (± 0.08)
Starved 24 hours (8)	0.40 (± 0.15)	0.35 (± 0.05)	0.57 (± 0.17)	0.24 (± 0.02)	0.18 (± 0.01)
Starved 48 hours (10)	0.30 (± 0.082)	0.31 (± 0.12)	0.52 (± 0.21)	0.20 (± 0.02)	0.18 (± 0.07)

B. Treated with phloridzin.

Starved 24 hours (10)	0.23 (± 0.034)	0.27 (± 0.1)	0.67 (± 0.17)	0.19 (± 0.05)	0.18 (± 0.04)
Starved 48 hours (9)	0.18 (± 0.07)	0.19 (± 0.04)	0.52 (± 0.15)	0.20 (± 0.06)	0.16 (± 0.05)

It is clear that 24 hours' and still more 48 hours' starvation greatly reduces liver- and muscle-glycogen. Our results agree with those of Cori and Cori [1928] but differ from those of Barbour *et al.* [1927] who found more glycogen in the liver after 48 than after 24 hours' starvation. Our absolute figures for the starved liver are also higher, but this we are inclined to attribute to the use of 80 % alcohol. We can find no previous determinations of the glycogen of the heart, stomach or kidney of the rat with which to compare our figures. The periods of starvation employed by us make no difference to the glycogen content of the heart, stomach or kidney, a new observation in the latter two organs. As regards the heart Jensen [1902] also found that prolonged starvation of two dogs for 15-17 days did not reduce heart-glycogen, although the latter was found to be very low in one dog after 19 days. This animal, however, was moribund.

Our periods of starvation were not prolonged, but were long enough to show that these three organs are much more tenacious of their glycogen than the liver and skeletal muscle. It seems certain that in the heart, kidney and stomach the formation and breakdown of glycogen differ from these processes

both in the liver and skeletal muscles and are independent of factors which profoundly affect the latter organs.

(2) *The effects of phloridzin.* (a) + 24 hours' starvation; (b) + 48 hours' starvation; Table II B.

The action of this drug in depleting liver and muscles of glycogen is well-known and has been reported in a voluminous literature. Its action on other organs has been very incompletely studied and the comprehensive reviews of Lusk [1912] and Nash [1927] do not mention it. Junkersdorf [1923] made a few determinations of heart- and kidney-glycogen in starved phloridzinised dogs and found the heart-glycogen raised if anything above the normal level. He does not, however, discuss its significance. 20 mg. (0.2 g. per kg.) of phloridzin was administered subcutaneously in olive oil on three consecutive days. The animals were killed 4–6 hours after the last dose and after 24 or 48 hours' starvation. They became glycosuric after the first dose and soon developed ketonuria. Phloridzin was also administered to a few rats for periods of from 10–14 days with no different result. The blood-sugars of these rats were at low normal levels (0.08–0.1 %).

The averages of our results (Table II B) agree with those of others in showing that phloridzin reduces the glycogen of the liver and muscles slightly below starvation levels. Phloridzin, however, has no effect on the glycogen of the heart, stomach or kidney, which confirms and extends Junkersdorf's experiments.

We do not think the slightly higher glycogen in the phloridzinised rat's heart has any significance. The failure of phloridzin to affect the glycogen of the heart, stomach and kidney is further evidence that the factors controlling the glycogen metabolism of these organs are not the same as those controlling that of the liver or skeletal muscles.

(3) *The effect of thyroid extract.*

Cramer and Krause [1913] first showed that the feeding of thyroid gland reduced the glycogen in the liver of cats and rats to mere traces. This has been confirmed by Kuriyama [1917] and Burn and Marks [1925]. We can find no record of its effect on the glycogen of other organs.

The rats were fed with crushed thyroid tablets (B. and W.) given in melted butter added to their usual diets. In this form the thyroid was well taken. They required 2–3 gr. (0.12–0.19 g.) daily per 100 g. body weight to make them lose weight satisfactorily. Rats therefore require enormous doses compared with rabbits and man. A comparable dose in man would be 2000 gr. daily. The glycogen effects are obtained long before any loss of weight becomes manifest. Table III shows the glycogen distribution in 24 hours' starved hyperthyroid rats, and Table IV that in "fully fed" hyperthyroid animals. The controls are Table II A. Some results require special mention.

(a) The hyperthyroid 24 hours' starved liver contains less glycogen than its control and no accumulation of glycogen takes place in the hyperthyroid liver even when "fully fed." These facts were already known and form a

Table III. *Thyroid-fed rats: starved 24 hours.*

Rat	Liver	Muscle	Heart	Stomach	Kidney	Blood-sugar
1	0.16	0.23	0.04*	0.11	0.12	—
2	0.42	0.52	0.17	0.17	0.16	—
3	0.29	Lost	0.18	0.14	0.14	0.118
4	0.22	0.19	0.23	0.09	0.14	0.327
5	0.25	0.21	0.13	0.22	0.14	—
6	0.21	0.12	0.15	0.20	0.14	0.000
7	0.23	0.24	0.24	0.16	0.12	0.056
8	0.18	0.30	0.16	0.14	0.09	0.093
Average	0.25	0.26	0.18	0.15	0.13	—

* Omitted from the average.

Table IV. *Thyroid-fed rats: fully fed.*

Rat	Liver	Muscle	Heart	Stomach	Kidney	Blood-sugar
1	0.26	0.54	0.17	0.39	0.15	0.129
2	0.32	0.53	0.22	0.19	0.17	0.130
3	0.43	0.79	0.58*	0.24	0.24	0.142
4	0.36	0.68	0.22	0.12	0.13	0.126
5	0.21	0.64	0.23	0.14	0.13	0.137
6	0.29	1.00	0.15	0.29	0.18	0.153
7	0.37	1.03	0.12	0.33	0.19	0.139
8	0.35	0.76	0.19	0.24	0.17	0.126
Average	0.32	0.75	0.18	0.24	0.17	—

* Omitted from the average.

strong argument in Cramer's contention that liver-glycogen is not formed directly from ingested carbohydrate, but is a specific secretion of the liver from protein and probably fat. We hope to take this matter up in a further communication.

(b) The hyperthyroid 24 hours' starved skeletal muscle contains on the average a little less glycogen than its controls. If this has any significance it may reasonably be explained as the result of the raised basal metabolic rate. The hyperthyroid fully fed muscles on the other hand contain not only as much, but perhaps more glycogen than normal fully fed animals. Bösl [1928] found no reduction in the muscle-glycogen of guinea-pigs injected with thyroxine. It is thus evident that hyperthyroidism has no deleterious effect upon glycogen storage in the muscles and that the glycogen not stored in the hyperthyroid liver may be found in the muscles, due allowance being made for the increased metabolism.

(c) The hyperthyroid heart "24 hours' starved" contains very much reduced amounts of glycogen, and these amounts are not increased after food. This effect of thyroid is a very striking one. Is it due to (1) a specific toxic action; (2) overwork; (3) lack of adequate carbohydrate supply? The last is scarcely possible in view of the glycogen storage in these animals' skeletal muscles. We do not think overwork is the reason because adrenaline produces a faster heart rate than thyroid without reducing the glycogen. Also Visscher and Mulder [1930] have recently shown in the heart-lung preparation that work does not reduce heart-glycogen. We are inclined to think therefore that

in this effect thyroid is a specific cardiac toxin. It is interesting to compare the disastrous effects of hyperthyroidism on the human heart.

Histological examination of the hearts of five hyperthyroid rats which died during hot weather in 1930 showed degenerative changes of varying degree, cloudy swelling and poor striation in both auricles and ventricles. In one striation was almost invisible and patches of necrosis with early cellular infiltration were seen. We are indebted to Dr Terence East for this report.

The blood-sugars of the starved hyperthyroid rats show some remarkable variations for which we can offer no explanation. There is no reason, however, to suspect their accuracy.

(d) The glycogen in the stomach and kidney of the 24 hours' starved hyperthyroid rats shows some reduction compared with the normal controls. We are inclined to think that the reduction has significance and is produced by the increased metabolism. The glycogen returns to normal after food.

(e) *Primary effect on the heart.* After one week (sometimes after 3 days) of thyroid administration the influence of the drug on the glycogen metabolism of the liver and heart has reached its maximum. We have carried out 20 experiments upon animals after from 3 to 6 days' administration to determine whether the heart or the liver was the first to be affected. Table V shows the

Table V. *Animals "fully fed." Early stages of hyperthyroidism.*

Glycogen %		Days' thyroid administration
Liver	Heart	
1.79	0.19	3
1.17	0.36	3
0.68	0.13	4
1.30	0.15	4
0.80	0.14	4
0.86	0.17	4
0.51	0.08	4
0.50	0.14	4
1.40	0.20	4
0.95	0.20	4
0.48	0.12	4
1.70	0.14	6
1.07	0.32	3
0.87	0.15	3
1.90	0.20	6
Average	1.07	0.17

results excluding (a) one whose liver- and heart-glycogen fell within the average deviation from the normal means; (b) four animals whose liver- and heart-glycogen fell within the average deviations from the fully fed hyperthyroid means. The table shows that full glycogen depletion of the heart may be obtained when the liver is almost unaffected. No contradictory results were obtained in which the liver was depleted before the heart. The effects of hyperthyroidism emphasise once more that the factors controlling glycogen metabolism may differ from organ to organ.

(4) *The effect of adrenaline.*

This has been made a very special study by Cori and Cori [1928, 1, 2, 3; 1929; 1930] so far as the liver and muscles of the rat are concerned. No other organs have so far as we are aware been investigated. Table VI B shows our experimental results and Table VI A the controls. The mean of our results

Table VI.

Rats, treatment and number	Mean % of glycogen and average deviation from mean						Blood-sugar mg. per 100 cc.
	Liver	Muscle	Heart	Stomach	Kidney	Spleen	
A. Normal (9)	0.28 (± 0.06)	0.30 (± 0.06)	0.64 (± 0.14)	0.18 (± 0.03)	0.15 (± 0.01)*	0.24 (± 0.05)†	0.085 (± 0.011)‡
B. 24 hours' starved; 0.05 mg. adrenaline per 100 g. 2 hours before death (9)	0.33 (± 0.08)	0.19 (± 0.05)	0.58 (± 0.20)	0.20 (± 0.03)	0.14 (± 0.01)	0.23 (± 0.07)†	0.205 (± 0.071)
C. 24 hours' starved; 0.1-0.2 units of insulin per 100 g. (8)	0.21 (± 0.02)	0.20 (± 0.05)	0.53 (± 0.09)	0.16 (± 0.025)	0.13 (± 0.02)	0.22 (± 0.05)†	0.074 (± 0.011)
D. 24 hours' starved; 0.4-3.0 units of pituitary extract 2-3½ hours before death (8)	0.24 (± 0.03)	0.29 (± 0.08)	0.60 (± 0.08)	0.21 (± 0.04)†	0.13 (± 0.01)	0.18 (± 0.025)	0.082 (± 0.015)

* 8 animals.

† 7 animals.

‡ 6 animals.

confirms Cori's contention that in the fasting rat adrenaline increases the liver-glycogen and decreases the muscle-glycogen. The glycogen of all the other organs including the heart is not significantly changed by this drug. The mean of the adrenalinised heart is slightly lower, but this is due to two very low results. The remainder are fully as high as the normals. The blood-sugar is raised in all cases by adrenaline, but this has not led to an increased deposition of glycogen in any of the organs.

(5) *The effect of insulin.*

Insulin was given in subconvulsive doses of 0.1-0.2 units per 100 g. and the animals were killed 2 hours later. The results in Table VI C show no appreciable reduction in the glycogen of heart, stomach, kidney or spleen. The liver- and muscle-glycogen are both slightly reduced.

(6) *The effect of pituitrin.*

The results are shown in Table VI D. The glycogen of all the organs, and the blood-sugar, lie very close to the controls, and pituitrin evidently has no effect on glycogen metabolism under these experimental conditions.

(7) *Other organs.*

We have not yet studied the glycogen content of other organs, but have made a few isolated estimations which give the following averages:

	%	Animals
Uterus	0.35	8
Brain	0.15	4
Testicle	0.09	2

GENERAL DISCUSSION.

The exact chemical nature of glycogen being unknown, any speculation on the possibility of its chemical modification from organ to organ is useless.

Physiologically, however, it is clear that the glycogens of various tissues behave quite differently. This has been accepted as regards liver- and skeletal muscle-glycogen whose products of disintegration, glucose and lactic acid respectively, and whose functions are so entirely different. Isolated observations on heart-glycogen by Cruickshank [1913] and on blood- and general tissue-glycogen by many workers [see Allen, 1913] have shown that it may behave quite differently from liver- and muscle-glycogen in these organs in diabetes. Thus while the diabetic liver may contain only traces of glycogen, and the muscles less than normal, the heart, blood and kidney may contain increased amounts. Our results also show how glycogen metabolism may differ from organ to organ. It is interesting to note the narrow range of glycogen concentration (0.15-0.25 %) in such organs as the kidney, spleen and brain. Also in our experiments various agents have reduced glycogen of the liver, muscle and heart, organs with a higher normal content and perhaps a specialised glycogen function, to similar concentrations. These facts suggest the presence in all organs of a residual non-specific glycogen, which the cells always retain, and which is quite different in physiological behaviour and perhaps chemically from the larger and more variable glycogen content of the liver, muscles and heart. These facts should be taken into account in formulating any complete theory of glycogen metabolism.

SUMMARY.

Table VII contains a summary of the results.

Table VII. *Summary.*

	Mean glycogen content of organs of rats.							No. of animals
	Liver	Muscle	Heart	Stomach	Kidney	Spleen		
Fully fed	2.30	0.54	0.52	0.21	0.18	0.21		12
Starved 24 hours	0.40	0.35	0.57	0.24	0.18	—		8
Starved 48 hours	0.30	0.31	0.52	0.20	0.18	—		8
Phloridzin. Starved 24 hours	0.23	0.27	0.67	0.19	0.18	—		8
Starved 48 hours	0.18	0.19	0.52	0.20	0.16	—		8
Thyroid. Starved 24 hours	0.25	0.26	0.18	0.15	0.13	—		8
Fully fed	0.32	0.75	0.18	0.24	0.17	0.21		8
3-6 days thyroid	1.07	—	0.17	—	—	—		15
New controls. Starved 24 hours	0.28	0.30	0.64	0.18	0.15	0.23		9
Adrenaline. Starved 24 hours	0.33	0.19	0.56	0.20	0.14	0.23		9
Insulin. Starved 24 hours	0.21	0.20	0.53	0.16	0.13	0.22		8
Pituitrin	0.24	0.29	0.60	0.21	0.13	0.18		8

1. Starvation does not reduce the glycogen of the heart, kidney or stomach.
2. Phloridzin reduces the glycogen of the liver and muscles slightly below starvation level, but has no effect on that of the heart, stomach or kidney.
3. Thyroid extract by mouth reduces very greatly the glycogen of the heart. The hyperthyroid heart is unable to replenish its glycogen stores even after high carbohydrate feeding. After 24 hours' starvation the hyperthyroid liver contains only slightly less glycogen than the control, but has completely

lost the normal ability to store glycogen after carbohydrate food. The hyperthyroid 24 hours' starved muscle contains slightly less glycogen than the normal, but storage is quite normal after carbohydrate ingestion.

The heart-glycogen is the first to be depleted by thyroid¹.

4. Adrenaline increases the liver-glycogen and decreases the muscle-glycogen of starved animals. It has no effect on the glycogen of other organs.

5. Insulin has no effect on the glycogen of heart, stomach or kidney. It reduces liver- and muscle-glycogen slightly.

6. Pituitrin has no effect on the glycogen of the fasting rat.

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REFERENCES.

- Allen (1913). Glycosuria and diabetes (pp. 97-103). (Harvard Univ. Press.)
Barbour, Chaikoff, Macleod and Orr (1927). *Amer. J. Physiol.* **80**, 243.
Bösl (1928). *Biochem. Z.* **202**, 299.
Burn and Marks (1925). *J. Physiol.* **60**, 131.
Cori and Cori (1928, 1). *J. Biol. Chem.* **79**, 309.
----- (1928, 2). *J. Biol. Chem.* **79**, 321.
----- (1928, 3). *J. Biol. Chem.* **79**, 343.
----- (1929). *J. Biol. Chem.* **84**, 683.
----- (1930). *J. Biol. Chem.* **86**, 375.
Cramer and Krause (1913). *Proc. Roy. Soc. Lond. B* **86**, 550.
Cruickshank (1913). *J. Physiol.* **47**, 1.
Holmes (1929). *Biochem. J.* **23**, 738.
Jensen (1902). *Z. physiol. Chem.* **35**, 514.
Junkersdorf (1923). *Pflüger's Arch.* **200**, 443.
Kuriyama (1917). *Amer. J. Physiol.* **43**, 481.
Lusk (1912). *Ergebn. Physiol.* **13**, 315.
Nash (1927). *Physiol. Rev.* **1**, 385.
Visscher and Mulder (1930). *Amer. J. Physiol.* **94**, 630.

¹ Note added 14. iv. 31. While this paper was in the Press, we have seen a recent article confirming this by Abderhalden and Wertheimer (1930), *Z. ges. exp. Med.* **72**, 472.

LXVII. METABOLISM IN SCURVY.

III. THE ABSORPTION AND RETENTION OF CALCIUM AND PHOSPHORUS BY GUINEA-PIGS.

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IN the last communication of this series, in which the mode of action of the antiscorbutic factor on the animal organism is being studied, it was shown that the pathological changes in the guinea-pig which form the syndrome of scurvy were not preceded by any disturbance in the absorption or in the retention of nitrogen [Shipp and Zilva, 1928]. This report deals with the metabolism of calcium and phosphorus by normal and scorbutic guinea-pigs.

It has already been pointed out by Shipp and Zilva that, since the macroscopic and, more so, the microscopic lesions of scurvy are observed soon after guinea-pigs are placed on a scorbutic diet, any abnormality in the metabolic functions in these animals which may be associated with the production of the disease ought to become manifest in the early stages of the experiment at a time when the animals are consuming adequate quantities of the diet. Changes which take place in the metabolism in later stages when the disease is severe and the animals are declining owing to insufficient consumption of food cannot be considered specific of scurvy. As microscopic scorbutic lesions can be established approximately 8 days after the guinea-pigs have been placed on the scorbutic diet [Zilva and Wells, 1919], it is evident that if the onset of scurvy be subsequent to any specific metabolic disturbance, this latter change will also develop fairly early. Furthermore, its manifestation may not be gradual but rapid, a circumstance which makes it advisable and even necessary to observe the daily intake and output of the elements studied, in order to establish whether, if such disturbance takes place, it precedes the development of the disease or is a consequence of it. The experimental scheme adopted was, therefore, similar to that previously employed by Shipp and Zilva in which the above precautions were taken and in which the retention and absorption of the two elements were also studied up to an advanced stage of experimental scurvy. In addition the calcium and phosphorus content of the blood of scorbutic guinea-pigs at various stages of the disease and of normal guinea-pigs was determined.

¹ Member of the Scientific Staff, Medical Research Council, working with grants for assistance and expenses.

EXPERIMENTAL.

Technique.

The experimental procedure adopted in this investigation was the same as that used by Shipp and Zilva [1928]. The following minor modifications were, however, introduced to suit the requirements of the present work. (1) Instead of preparing a stock solution of milk, sufficient for several days, about 5 g. of dried milk powder were weighed out each day, dissolved in 15 cc. of distilled water and dosed to each guinea-pig. In addition 25 cc. of distilled water which was also offered daily was usually consumed. (2) No acid was placed in the urine collecting vessel, but the cage, funnel, trap and receiving jars were rinsed out each morning with 0.5 N HCl, after the careful removal of all faeces, and the washings added to the collected urine.

The calcium in the urine, faeces and blood was estimated by the Kramer and Tisdal method [1921] and the phosphorus by Martland and Robison's modification [1924] of the Briggs [1922] method.

DISCUSSION OF RESULTS.

The retention and absorption of calcium and phosphorus by protected and scorbutic guinea-pigs.

Exp. 1. This experiment (Table I) was carried out with the view of studying the metabolism of calcium and phosphorus in the protected guinea-pig and, except for the longer duration, it is comparable with the preliminary periods of *Exp. 2* when the animals were fed on a diet of basal mixture, autoclaved milk and 5 cc. of decitrated lemon juice daily (Period 1, Tables II and III). The consumption of the basal diet during these preliminary periods was rather below that consumed by guinea-pigs kept in ordinary cages under similar conditions, and, as would be expected, the growth of these animals was less than normal. Apart from this the animals appeared perfectly healthy and active. The average total intake of calcium and phosphorus varied in the three cases and it will be seen that there is a fairly marked variation in the degree of absorption and retention of both elements.

Exp. 2. In this experiment (Guinea-pig 6, Table II) the metabolism of calcium and phosphorus was followed from day to day, both during the preliminary and scorbutic periods. In the preliminary period, although the animal did not show a net gain in weight its consumption of food and general condition of health were those of a protected guinea-pig. In the second period when the administration of decitrated lemon juice was stopped the average excretion and balance were higher than in the preceding period. The urinary calcium was also rather higher than in the first period, but the phosphorus excreted by the kidneys remained of the same order.

Table I. *Guinea-pig No. 9.*

Date Feb. 1929	Weight of animal g.	Basal mixture of con- sumed g.	Total intake of phos- phorus mg.	Total intake of calcium mg.	Urinary phos- phorus mg.	Faecal phos- phorus mg.	Total output of phos- phorus mg.	Ab- sorbed phos- phorus mg.	Urinary calcium mg.	Faecal calcium mg.	Total output of calcium mg.	Ab- sorbed calcium mg.	Re- tained phos- phorus mg.	Re- tained calcium mg.	P re- tained P in- take	P re- tained P ab- sorbed	Ca re- tained Ca in- take	Ca re- tained Ca ab- sorbed	Ca re- tained P re- tained
Period 1. 13 days on basal mixture, autoclaved milk and 5 cc. deuterated lemon juice <i>per diem</i> :																			
5	280	12.28	175.9	259.1	66.2	55.3	121.5	120.6	66.1	63.2	129.3	195.9	+ 54.4	+ 129.8	0.3083	0.4511	0.5013	0.6626	2.368
6	277	13.10	177.1	314.0	86.7	33.0	119.7	144.1	96.9	29.5	126.4	284.5	+ 57.4	+ 187.6	0.3241	0.3983	0.5973	0.6606	3.268
7	279	14.69	201.9	261.4	81.9	54.2	136.1	147.7	100.3	52.7	153.0	208.7	+ 65.6	+ 108.4	0.3259	0.4452	0.4148	0.5194	1.647
8	283	16.88	222.8	299.9	55.8	57.3	113.1	165.5	116.0	65.3	181.3	234.6	- 100.7	+ 118.6	0.4925	0.6634	0.3956	0.5058	1.081
9	282	14.42	205.5	248.8	86.0	81.5	167.5	124.0	97.6	88.8	186.4	160.0	+ 38.0	+ 63.4	0.1807	0.3065	0.2502	0.3900	1.643
10	280	13.57	186.2	260.3	93.5	69.4	162.9	116.8	120.4	75.4	195.8	184.9	+ 23.3	+ 64.5	0.1251	0.1995	0.2478	0.3489	2.769
11	275	18.16	233.7	311.2	102.1	65.0	167.1	168.7	132.6	72.1	204.7	239.1	+ 66.6	+ 106.5	0.2849	0.3949	0.3423	0.4454	1.599
12	282	9.16	128.4	181.7	75.8	28.3	104.1	100.1	115.2	41.0	156.2	140.7	+ 21.3	+ 25.5	0.1888	0.2428	0.1402	0.1812	1.049
13	280	14.00	195.1	244.6	86.4	35.8	122.2	159.3	104.1	57.4	161.5	187.2	+ 72.9	+ 83.1	0.3741	0.4576	0.3397	0.4439	1.140
14	285	13.16	158.4	235.5	66.0	38.8	104.8	119.6	98.9	57.9	156.8	177.6	+ 53.6	+ 78.7	0.3385	0.4481	0.3345	0.4431	1.469
15	288	14.07	191.9	245.8	76.9	37.8	114.7	154.1	108.0	58.9	166.9	186.9	+ 77.2	+ 78.9	0.4021	0.5009	0.3214	0.4222	1.022
16	285	13.70	176.4	232.8	89.3	85.2	174.5	91.2	118.7	105.5	224.2	127.3	+ 1.9	+ 8.6	0.0107	0.0208	0.0369	0.0676	4.526
17	280	15.26	174.1	256.5	81.9	57.3	139.2	116.8	121.7	79.8	201.5	176.7	+ 34.8	- 55.0	0.1999	0.2980	0.2141	0.3113	1.581
Total	182.45	2427.4	3351.6	1048.5	698.9	698.9	1747.4	1728.5	1396.5	847.5	2244.0	2504.1	+ 680.0	+ 1107.6	0.2805	0.3928	0.3274	0.4424	1.63
Average		186.7	257.8	80.6	53.7	53.7	134.5	132.9	107.4	65.2	172.6	192.6		85.2					

"Absorbed" phosphorus (or calcium) = total intake of phosphorus (or calcium) less faecal phosphorus (or calcium).

"Retained" phosphorus (or calcium) = total intake of phosphorus (or calcium) less total output of phosphorus (or calcium).

Table II. *Guinea-pig No. 6.*

Date 1929 Mar.	Weight of animal g.	Basal mixture of con- sumed g.		Total intake of phos- phorus mg.		Total intake of calcium mg.		Urinary phos- phorus mg.		Faecal calcium mg.		Total output of calcium mg.		Re- tained phos- phorus mg.		Re- tained calcium mg.		P re- tained P in- P ab- sorbed take sorbed		Ca re- tained Ca ab- Ca ab- sorbed sorbed		Ca re- tained Ca in- Ca in- take sorbed				
		g.	g.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	
Period 1. 8 days on basal diet and autoclaved milk and 5 cc. decalcified lemon juice <i>per diem</i> :																										
14	337	20-01	240-7	354-4	90-6	68-4	157-0	174-3	131-7	94-5	226-2	259-9	+ 83-7	+ 128-2	0-3477	0-4803	0-3617	0-4933	0-3617	0-4803	0-3617	0-4933	0-3617	0-4933	0-3617	1-532
15	340	18-26	233-3	355-3	89-8	108-7	208-5	124-6	121-7	126-1	247-8	229-2	+ 24-8	+ 107-5	0-1064	0-1581	0-3026	0-4901	0-3026	0-1581	0-3026	0-4901	0-3026	0-4901	0-3026	1-535
16	330	20-02	246-8	351-3	85-8	105-6	191-4	141-2	115-7	123-4	239-1	227-9	+ 55-4	+ 112-2	0-2245	0-3823	0-3203	0-4923	0-3203	0-3823	0-3203	0-4923	0-3203	0-4923	0-3203	2-025
17	332	18-77	231-9	341-5	83-9	110-4	194-3	121-5	106-4	127-1	232-5	214-4	+ 37-6	+ 109-0	0-1623	0-3102	0-3192	0-5064	0-3192	0-3102	0-3192	0-5064	0-3192	0-5064	0-3192	2-896
18	337	13-85	215-1	302-5	62-9	72-4	135-3	142-7	82-2	82-1	164-3	220-4	+ 79-8	+ 138-2	0-3710	0-5593	0-3629	0-6270	0-3629	0-5593	0-3629	0-6270	0-3629	0-6270	0-3629	1-376
19	342	8-28	122-9	183-0	41-0	120-7	161-7	2-2	37-9	130-0	167-9	53-0	+ 38-8	+ 15-1	—	—	0-0824	0-2830	—	—	0-0824	0-2830	—	—	0-0824	0-2830
20	340	8-79	136-3	190-2	48-8	57-3	106-1	79-0	46-4	88-6	135-0	101-6	+ 30-2	+ 55-2	0-2216	0-3823	0-2970	0-5421	0-2970	0-3823	0-2970	0-5421	0-2970	0-5421	0-2970	1-828
21	335	16-95	218-3	316-9	55-2	75-3	130-5	143-0	98-4	97-7	196-1	219-2	+ 87-8	+ 120-8	0-4038	0-6296	0-3812	0-5511	0-4038	0-6296	0-3812	0-5511	0-4038	0-6296	0-3812	—
Total	124-93	1645-3	2395-1	568-0	716-8	1284-8	1284-8	928-5	739-4	869-5	1608-9	1525-6	+ 360-7	+ 786-2	0-2193	0-3886	0-3282	0-5159	0-2193	0-3886	0-3282	0-5159	0-2193	0-3886	0-3282	2-108
Average	15-62	205-7	299-4	71-0	89-6	160-6	160-6	116-1	92-4	108-7	201-1	190-7	+ 45-1	+ 98-3												
Period 2. 5 days on basal diet and autoclaved milk :																										
22	335	20-04	250-7	353-3	71-7	114-9	186-6	135-8	127-9	129-7	257-6	223-6	+ 64-1	+ 95-7	0-2558	0-4721	0-2708	0-4280	0-2708	0-4721	0-2708	0-4280	0-2708	0-4280	0-2708	1-493
23	343	21-33	257-6	378-1	76-1	184-2	290-3	73-4	114-9	186-5	301-4	191-6	+ 2-7	+ 76-7	—	—	0-2029	0-4004	0-2029	—	0-2029	—	0-2029	—	0-2029	—
24	340	20-64	252-4	359-9	97-6	144-4	242-0	108-0	127-8	146-2	274-0	213-7	+ 10-4	+ 85-9	0-0412	0-0943	0-2386	0-4020	0-2386	0-0943	0-2386	0-4020	0-2386	0-4020	0-2386	8-262
25	345	21-83	288-2	408-9	79-7	83-2	162-9	205-0	124-1	127-4	251-5	270-5	+ 125-3	+ 152-4	0-4347	0-6110	0-3772	0-5511	0-3772	0-6110	0-3772	0-5511	0-3772	0-5511	0-3772	1-354
26	346	22-29	285-8	395-0	69-8	99-7	169-5	186-1	107-6	184-0	291-6	211-0	+ 116-3	+ 103-4	0-4070	0-6250	0-2817	0-4900	0-2817	0-6250	0-2817	0-4900	0-2817	0-4900	0-2817	0-889
Total	108-13	1334-7	1890-2	394-9	626-4	1021-3	1021-3	708-3	602-3	773-8	1376-1	1116-4	+ 313-4	+ 514-1	0-2336	0-4432	0-2658	0-4606	0-2658	0-4432	0-2658	0-4606	0-2658	0-4606	0-2658	1-640
Average	21-23	266-9	378-0	79-0	125-3	204-3	204-3	141-7	120-5	154-8	255-2	223-3	+ 62-7	+ 102-8												
Period 3. 5 days on basal diet and autoclaved milk :																										
27	352	21-09	361-8	376-3	69-2	145-2	214-4	216-6	105-3	140-4	245-7	235-9	+ 147-4	+ 130-6	0-4074	0-6969	0-3469	0-5530	0-3469	0-6969	0-3469	0-5530	0-3469	0-5530	0-3469	0-886
28	354	18-84	223-8	368-2	65-6	49-3	114-9	184-5	108-3	108-3	215-2	260-9	+ 118-9	+ 154-0	0-5008	0-6446	0-4172	0-5902	0-4172	0-5008	0-4172	0-5902	0-4172	0-5902	0-4172	1-285
29	360	16-83	211-4	354-9	67-9	128-0	195-9	83-4	108-8	93-9	202-7	261-0	+ 15-5	+ 152-2	0-0733	0-1858	0-4286	0-5908	0-4286	0-1858	0-4286	0-5908	0-4286	0-5908	0-4286	9-819
30	362	19-93	288-1	336-4	70-9	100-6	171-5	137-5	119-9	99-1	219-0	237-3	+ 66-6	+ 117-4	0-2803	0-4844	0-3920	0-4948	0-3920	0-4844	0-3920	0-4948	0-3920	0-4948	0-3920	1-763
31	372	19-58	244-4	350-5	84-7	121-2	205-9	123-2	111-8	119-8	261-6	200-7	+ 38-5	+ 88-9	0-1575	0-3125	0-2536	0-4430	0-2536	0-3125	0-2536	0-4430	0-2536	0-4430	0-2536	2-310
Total	96-87	1289-5	1787-3	358-3	544-3	902-6	902-6	745-2	582-7	591-5	1144-2	1195-8	+ 386-9	+ 643-1	0-2999	0-5192	0-3599	0-5378	0-2999	0-5192	0-3599	0-5378	0-2999	0-5192	0-3599	1-083
Average	19-37	257-9	359-5	71-7	106-9	180-5	180-5	149-0	110-5	108-3	228-8	239-2	+ 77-4	+ 128-6												

Table III. *Guinea-pig No. 11.*

Date 1929 May	Weight of animal g.	Basal mixture con- sumed g.	Total intake of phos- phorus mg.	Total intake of calcium mg.	Urinary phos- phorus mg.	Faecal phos- phorus mg.	Ab- sorbed phos- phorus mg.	Re- tained phos- phorus mg.	Ab- sorbed calcium mg.	Re- tained calcium mg.	P re- tained P in- take	P re- tained P ab- sorbed	Ca re- tained Ca in- take	Ca re- tained Ca ab- sorbed	Ca re- tained P re- tained
Period 1. 7 days on basal mixture, autoclaved milk and 5 cc. dehydrated lemon juice:															
6	300	14.47	196.1	263.3	73.5	58.1	138.0		139.7	+ 64.5	0.3390	0.4774	0.07861	0.1480	0.3201
7	300	14.08	202.0	263.8	72.9	80.8	121.2		152.4	+ 48.4	0.2394	0.3996	0.1365	0.2523	0.8285
8	287	17.33	223.2	323.8	74.6	82.2	141.0		200.9	+ 66.4	0.2975	0.4743	0.1730	0.2786	0.8433
9	318	18.91	225.3	357.1	82.3	98.1	127.2		199.4	+ 44.8	0.1988	0.3572	0.1897	0.3405	1.4860
10	321	17.35	240.7	337.9	77.5	101.6	139.1		192.0	+ 61.6	0.2560	0.4439	0.1344	0.2634	0.7528
11	323	17.08	246.7	341.4	83.3	102.8	143.9		181.9	+ 60.5	0.2453	0.4206	0.1479	0.2776	0.8347
12	320	16.53	229.2	338.2	92.4	84.9	144.3		205.0	+ 51.8	0.2260	0.3589	0.1782	0.2936	1.1620
Total		115.75	1563.2	2255.5	556.4	608.5	954.7		1271.3	+ 398.0	0.2554	0.4172	0.1511	0.2681	0.8559
Average		16.54	223.2	322.2	79.5	86.9	136.4		181.6	+ 56.7					
Period 2. 5 days on basal mixture and autoclaved milk, no antiscorbutic:															
13	322	18.95	255.9	365.5	74.6	139.8	116.1		188.4	+ 41.5	0.1622	0.3574	0.2092	0.4050	1.8390
14	320	15.35	233.7	316.6	74.6	96.7	137.0		183.8	+ 62.4	0.2670	0.4565	0.2084	0.3590	1.0580
15	320	14.43	196.9	281.5	53.6	135.9	61.0		194.3	+ 7.4	0.0376	0.1211	0.0944	0.1332	3.4960
16	310	19.02	227.1	332.0	62.5	138.9	88.2		171.7	+ 25.7	0.1132	0.2914	0.1738	0.3359	2.2460
17	310	19.01	244.1	336.4	82.3	154.3	89.8		169.2	+ 7.5	0.0307	0.0816	0.1127	0.2240	5.0530
Total		86.79	1157.7	1632.0	347.6	605.6	492.1		817.4	+ 144.5	0.1248	0.2936	0.1615	0.3228	1.8220
Average		17.36	231.5	326.5	69.5	133.1	98.4		163.5	+ 28.9					
Period 3. 6 days on basal mixture and autoclaved milk, no antiscorbutic:															
18	313	17.99	247.6	338.5	72.6	134.6	113.0		181.5	+ 40.4	0.1631	0.3576	0.1630	0.3597	1.6160
19	310	17.96	223.0	310.8	73.8	135.0	88.0		155.8	+ 14.2	0.0637	0.1612	0.1345	0.2683	2.9400
20	312	19.82	240.8	326.4	76.9	123.8	116.8		182.5	+ 38.9	0.1533	0.3159	0.1841	0.3284	1.6290
21	313	18.80	224.5	304.5	73.8	124.6	99.9		171.5	+ 26.1	0.1160	0.2612	0.1351	0.2456	1.6100
22	315	17.72	213.2	318.1	66.9	130.1	83.1		160.1	+ 16.2	0.0760	0.1949	0.1211	0.2400	2.3770
23	320	16.72	203.5	335.1	63.9	106.0	97.5		198.0	+ 34.6	0.1700	0.3557	0.2830	0.4787	2.7390
Total		108.01	1352.4	1933.4	426.9	754.1	598.3		1049.4	+ 171.4	0.1288	0.2865	0.1776	0.3271	2.0020
Average		14.00	225.4	322.2	71.2	125.7	99.7		174.9	+ 28.6					

Period 4. 5 days on basal mixture and autoclaved milk, no ascorbic:

24	323	18.40	210.3	344.4	73.0	142.4	67.9	135.5	171.0	173.4	- 5.1	+ 37.9	-	-	-	0.1100	0.2186	-
25	324	14.63	166.7	283.0	64.7	130.5	36.2	107.6	158.2	124.8	-28.5	+ 17.2	-	-	-	0.0765	0.1378	-
26	327	14.73	167.6	266.6	71.2	102.0	65.6	116.8	114.7	151.9	- 5.6	+ 35.1	-	-	-	0.1316	0.2311	-
27	328	12.09	144.1	212.7	63.1	100.9	43.2	85.5	120.2	92.5	-19.9	+ 7.0	-	-	-	0.3291	0.0756	-
28	320	10.36	132.6	234.0	60.2	75.7	56.9	92.5	84.3	149.7	- 4.3	+ 57.1	-	-	-	0.2440	0.3814	-
Total		70.21	821.3	1340.7	332.2	551.5	209.8	537.9	648.4	692.3	-63.4	+154.3	-	-	-	0.1156	0.2229	-
Average		14.04	164.3	268.1	66.4	110.3	54.0	107.6	120.7	138.5	-12.7	+ 30.9	-	-	-			-

Period 5. 5 days on basal mixture, autoclaved milk and 5 cc. decitrated lemon juice:

29	322	11.45	163.5	234.6	69.0	79.6	83.9	93.5	73.6	161.0	+13.8	+ 67.6	0.0638	0.1632	0.2822	0.2595	4.8210
30	317	12.14	178.4	251.0	74.5	76.7	101.7	108.3	99.9	151.1	-26.2	+ 42.8	0.1469	0.2577	0.1705	0.2832	1.6330
31	312	14.38	200.6	278.4	68.5	83.9	116.7	97.9	100.8	177.6	+51.2	+ 79.7	0.2496	0.4377	0.2864	0.4477	1.5570
June																	
1	318	15.11	203.0	286.1	67.6	122.1	80.9	101.1	150.3	135.8	+13.4	+ 34.7	0.0660	0.1657	0.1213	0.2555	2.5890
2	313	16.06	221.8	303.3	66.7	86.8	135.0	99.6	115.9	187.4	-68.3	+ 87.7	0.3080	0.5059	0.2892	0.4651	1.2770
Total		69.14	967.3	1353.4	346.3	449.1	518.2	500.4	540.5	812.9	+172.9	+312.5	0.1787	0.3336	0.2312	0.3844	1.8070
Average		13.83	193.5	270.7	69.3	89.8	103.6	100.1	108.1	162.6	+ 34.6	+ 62.5	-	-	-		

Period 6. 4 days on basal mixture, autoclaved milk and 5 cc. decitrated lemon juice:

3	322	16.88	222.5	352.2	71.7	91.7	130.8	100.9	124.4	227.8	+59.2	+ 99.9	0.2661	0.4307	0.2844	0.4386	1.6880
4	321	15.29	190.4	280.5	92.5	103.0	96.4	103.6	123.4	157.1	+ 3.9	+ 53.5	0.1956	0.4049	0.1907	0.3406	13.7200
5	321	17.41	228.9	333.2	61.9	119.9	109.0	99.0	122.6	210.6	+47.0	+111.6	0.2053	0.4312	0.3273	0.5299	2.3740
6	325	18.15	239.5	354.8	64.1	106.2	131.3	99.8	111.9	242.9	+67.2	-143.2	0.2806	0.5118	0.4635	0.5895	2.1250
Total		67.73	890.3	1320.7	290.2	422.8	467.5	403.3	482.3	838.4	+177.3	+408.2	0.1992	0.3793	0.3154	0.5029	2.3150
Average		16.93	222.6	330.2	72.5	105.7	116.9	100.8	120.6	209.6	+44.3	+102.1	-	-	-		

However, as far as one can judge from these figures and those of the following experiment, no striking deviation from the normal metabolism of calcium and phosphorus takes place during the first 10 days on a scorbutic diet. During the following two scorbutic periods (4 and 5) the intake of food diminished and the balance eventually fell in consequence, that of phosphorus actually becoming negative. The faecal phosphorus diminished in both periods whilst the calcium did so only during period 5. There was a decided drop in the urinary calcium and phosphorus during this period. These changes certainly show a deviation from the normal, but they are, presumably, not connected with the onset of scurvy but are the effect of a general metabolic disturbance produced by the disease. In the last period decitrated lemon juice was again given to the animal but, as may be seen from the figures, the disturbance in the metabolism was too advanced to be rectified during the 4 days of treatment.

If we now turn our attention to guinea-pig 11 (Table III) we see a picture similar to that presented by guinea-pig 6. In view of the observations previously made here to the contrary one cannot attach much importance to the slight average rise in the faecal, and fall in the urinary, calcium during periods 2 and 3. On the other hand, as in the case of guinea-pig 6, there was a marked rise in the faecal phosphorus during these periods. This observation was considered at the time the more noteworthy since there was not even an increased intake of this element during these periods. It will be seen that in the case of guinea-pig 11 there were only three scorbutic periods (15 days) and consequently the return to normal metabolism took place shortly after resuming the administration of the antiscorbutic dose.

Exp. 3. It was seen in *Exp. 2* that a rise in the faecal excretion of phosphorus by both guinea-pigs occurred during the first two scorbutic periods whilst the calcium was not affected. In guinea-pig 6 this could to some extent be explained by the increased food intake during the early days on the scorbutic diet. Such an increased consumption, however, did not, as already mentioned, occur in the case of guinea-pig 11. It is evident that if a raised faecal excretion of phosphorus were characteristic of the onset of scurvy it should be possible to repeat it without difficulty, since the production of experimental scurvy in young guinea-pigs is marked by little individual variation. The daily intake and faecal excretion of phosphorus were therefore studied in three guinea-pigs, but no rise in the faecal phosphorus could be observed. For the sake of economy of space the averages of the various periods are given in Tables IV, V and VI. It will be seen that in the case of guinea-pig 14 (Table IV) there is even a somewhat smaller ratio of faecal to consumed phosphorus during the first two scorbutic periods than in the preceding preliminary periods, whilst in guinea-pigs 17 and 19 there is no appreciable change to be observed in this ratio. It seems, therefore, highly improbable that an increased faecal excretion of phosphorus is primarily associated with the onset of scurvy in the guinea-pig and the rise observed in

Table IV. *Guinea-pig No. 14.*

Period		Change in weight g.	Total intake of P mg.	Total faecal P mg.	Average daily intake of P mg.	Average daily faecal P mg.	Faecal P intake of P
1	5 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	+ 15	831.30	494.21	166.26	98.84	0.5946
2	4 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	- 3	590.40	341.40	147.60	85.35	0.5782
3	5 days on basal diet and auto-claved milk	0	739.31	349.11	147.86	69.82	0.4720
4	4 days on basal diet and auto-claved milk	+ 16	871.07	414.75	217.77	103.69	0.4760
5	4 days on basal diet and auto-claved milk	+ 2	809.78	379.81	202.45	94.95	0.4689
6	5 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	+ 15	1075.09	624.68	215.02	124.94	0.5812
7	4 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	+ 10	921.59	495.30	230.40	123.83	0.5379
8	4 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	+ 10	957.26	604.80	239.32	151.20	0.6319
9	4 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	0	930.42	499.81	232.61	124.95	0.5374

Table V. *Guinea-pig No. 19.*

Period		Change in weight g.	Total intake of P mg.	Total faecal P mg.	Average daily intake of P mg.	Average daily faecal P mg.	Faecal P intake of P
1	5 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	+ 2	970.11	489.47	194.02	97.89	0.5046
2	4 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	+ 10	883.41	397.28	220.85	99.32	0.4504
3	5 days on basal diet and auto-claved milk	+ 23	925.60	454.50	185.12	90.90	0.4911
4	4 days on basal diet and auto-claved milk	+ 10	990.79	519.30	247.70	129.83	0.5242
5	4 days on basal diet and auto-claved milk	0	724.33	365.78	181.08	91.45	0.5049
6	5 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	+ 13	1028.25	544.71	205.65	108.94	0.5297
7	4 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	+ 7	981.77	447.61	245.44	111.90	0.4557
8	4 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	+ 20	1074.75	582.70	268.69	145.68	0.5423
9	4 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	+ 10	1033.62	535.50	258.41	133.88	0.5177

Table VI. *Guinea-pig No. 17.*

Period	Change in weight g.	Total intake of P mg.	Total faecal P mg.	Average daily intake of P mg.	Average daily faecal P mg.	Faecal P Intake of P
1 5 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	- 8	778.21	397.09	155.64	79.42	0.5104
2 4 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	+ 3	814.00	423.83	203.50	105.96	0.5206
3 4 days on basal diet and auto-claved milk	+ 8	725.54	381.91	181.39	95.48	0.5264
4 4 days on basal diet and auto-claved milk	+ 12	861.93	455.91	215.48	113.98	0.5291
5 4 days on basal diet and auto-claved milk	- 8	731.71	379.07	182.93	94.77	0.5180
6 5 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	+ 5	883.30	413.55	176.66	82.71	0.4680
7 4 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	+ 17	800.44	393.47	200.11	98.37	0.4919
8 6 days on basal diet, auto-claved milk and 5 cc. of decitrated lemon juice <i>per diem</i>	+ 16	1407.86	571.13	234.64	95.19	0.4057

Exp. 2 must have been due to a cause other than the consumption of the scorbutic diet.

The calcium and phosphorus content of the blood of protected and scorbutic guinea-pigs.

Before proceeding to study the calcium and phosphorus content of the blood it was essential to ascertain how the composition of the blood in this respect was affected by the time elapsing between the animal's last meal and the collection of the blood for analytical purposes. In the case of a diet offered *ad lib.* it is impossible to know when the animal has its last feed and therefore if the calcium and phosphorus of the blood were influenced by the time of consumption of the last meal it would obviously vitiate any comparative results unless precautions were taken.

Table VII.

Guinea-pigs nos.	Weight g.	Diet	Remarks	Phosphorus whole blood mg./100 cc.
22	375	Oats, bran and cabbage <i>ad lib.</i>	Starved 18 hrs. prior to bleeding	36.35
36	380	"	"	31.62
27, 28	247, 250	"	"	34.61
39, 38	275, 270	Basal diet, <i>ad lib.</i> 40 cc. milk and 5 cc. decitrated lemon juice	"	41.36
31, 37	287, 280	"	Not starved	39.83
30, 29	275, 300	"	"	47.92

All animals bled by severing the jugular vein and carotid artery after anaesthesia.

Exp. 4. All the details of this experiment are summarised in Tables VII and VIII. The results make it plain that although there is a considerable individual variation there is no indication that the blood-calcium and blood-phosphorus of guinea-pigs fed on the basal diet and receiving their anti-scorbutic in the form of decitrated lemon juice are influenced by the length of the interval between the last meal and the collection of the blood.

Table VIII.

Guinea-pigs nos.	Weight g.	Diet	Remarks	Calcium whole blood mg./100 cc.
168, 178	280, 235	Basal, 40 cc. milk and 5 cc. decitrated lemon juice	Starved 18 hrs. Bled 1½ hrs. after giving food	9.149
175, 174	275, 277	"	"	9.897
169, 164	247, 290	"	Starved 18 hrs. Bled 5 hrs. after giving food	9.623
173, 163	240, 280	"	"	8.292
165, 161	245, 250	"	Starved 18 hrs. prior to bleeding	9.942
172, 170	245, 265	"	"	9.813
160, 166	250, 255	"	Not starved	9.806
177, 179	237, 255	"	"	11.44
181, 185	260, 285	"	"	8.515
189, 190	280, 265	"	"	9.811
202, 203	275, 330	Basal, 40 cc. milk and 10 cc. decitrated lemon juice	"	8.974
198, 193	295, 315	"	"	8.855

All animals killed by cutting the jugular vein and carotid artery without anaesthesia.

Exp. 5. A number of guinea-pigs were then fed on the basal diet and 5 cc. of decitrated lemon juice *per diem* for about 16 days. Some were then killed by bleeding whilst others were kept on the same basal diet but without the antiscorbutic dose for further periods of 5, 8, 10, 15 and 20 days respectively before being bled. As will be seen from Table IX no change in the

Table IX.

Guinea-pigs nos.	Weight g.	Diet	No. of days on scorbutic diet	Phosphorus whole blood mg./100 cc.
29, 30	275, 300	Basal, autoclaved milk and 5 cc. decitrated lemon juice	0	47.92
31, 37	280, 287	"	0	39.85
38, 39	275, 300	"	0	41.36
40, 49	305, 325	Basal and milk	5	40.86
50, 51	326, 302	"	5	44.29
41, 42	282, 252	"	8	34.50
43, 44	245, 325	"	10	36.81
53, 55	255, 265	"	10	41.91
46, 48	215, 230	"	15	33.38
56	335	"	15	49.90
45, 47	255, 227	"	20	37.57
52, 57	299, 318	"	20	43.19

All animals killed by cutting the jugular vein and carotid artery after anaesthesia.

total phosphorus content of the blood was observed. As before great individual variations exist which cannot be traced to any change in diet.

Exp. 6. In Exps. 4 and 5 the guinea-pigs were anaesthetised prior to being bled. It was therefore decided to determine whether the elimination of the anaesthetic would bring about smaller individual variations in the blood-phosphorus. Table X gives the results of an experiment in which the blood

Table X.

Guinea-pigs nos.	Weight g.	Diet	No. of days on scorbutic diet	Calcium whole blood mg./100 cc.	Phosphorus whole blood mg./100 cc.
84, 87, 93	230, 280, 250	Basal and 5 cc. decitrated lemon juice	0	13.50	38.10
85, 89, 94	268, 282, 264	"	0	12.20	36.16
90, 91, 92	277, 303, 282	"	0	15.37	32.20
83, 86, 88	262, 270, 277	"	0	15.32	33.95
61	300	"	0	15.47	—
60	290	"	0	15.38	—
62, 63	270, 320	Basal only	5	12.59	40.30
74, 75	300, 320	"	5	11.28	40.60
64, 65	322, 365	"	10	10.98	43.60
76, 78	305, 320	"	10	11.22	43.65
67, 68	315, 325	"	15	10.48	42.00
77, 80	270, 300	"	15	12.84	36.76
70, 71	325, 315	"	20	13.90	41.75
81, 82	340, 285	"	20	14.42	41.55

All animals bled by severing the jugular vein and carotid artery without anaesthesia.

was collected without previously anaesthetising the animal. The variations in the blood-phosphorus were, however, still marked (Table X) but a somewhat lower content of calcium and higher average content of phosphorus were found in the whole blood during the scorbutic period. As the subsequent experiments will show, this was not due to the absence of the antiscorbutic factor.

Exp. 7. The effect of increasing the antiscorbutic dose was next investigated. The animals were given 10 cc. of decitrated lemon juice daily instead of 5 cc. as in the previous experiments. From Table XI it is seen that the

Table XI.

Guinea-pigs nos.	Weight g.	Calcium whole blood mg./100 cc.	Phosphorus whole blood mg./100 cc.
98	315	9.482	37.20
95	315	9.988	40.25
102	332	8.410	40.98
96	320	9.114	42.01
101	325	8.110	41.51
99	322	8.023	37.96
107	285	9.471	—

All animals bled by cutting the jugular vein and carotid artery without anaesthesia.

Diet—basal mixture, autoclaved milk and daily dose of 10 cc. decitrated lemon juice.

blood-phosphorus of guinea-pigs receiving 10 cc. of decitrated lemon juice daily is of the same order as that of the scorbutic animals in Exps. 5 and 6,

(Tables IX and X) whilst the blood-calcium is lower than that of the scorbutic and protected animals previously examined in Exp. 6 (Table X).

That this difference in the blood-calcium is not due to the higher anti-scorbutic dose is seen from the following experiment. A number of guinea-pigs were fed for about two weeks on the basal diet and autoclaved milk, one-half of them receiving 5 cc. and the remainder 10 cc. of decitrated lemon juice *per diem*. The antiscorbutic dose was then discontinued in both cases and the animals were killed after subsisting on the basal diet alone for 5, 8 and 10 days. The results (Tables XII and XIII) also show considerable individual variation but again give no indication of any alteration in the calcium and phosphorus content of the blood which can be traced to the exclusion of vitamin C from the diet.

Table XII.

Guinea-pigs nos.	Weight g.	Diet	No. of days on scorbutic diet	Calcium whole blood mg./100 cc.
160, 166	259, 255	Basal, autoclaved milk and 5 cc. decitrated lemon juice	0	9.806
177, 179	237, 255	"	0	11.44
181, 185	260, 285	"	0	8.515
189, 190	280, 265	"	0	9.811
202, 203	275, 330	Basal, autoclaved milk and 10 cc. decitrated lemon juice	0	8.974
198, 193	295, 315	"	0	8.855
183, 186	335, 280	Basal and autoclaved milk	5	8.845
182, 187	270, 307	"	5	9.770
194, 199	255, 285	"	5	8.800
195	315	"	5	9.603
188, 191	340, 295	"	10	9.705
180	325	"	10	12.34
197, 201	275, 325	"	10	9.301
176, 200	240, 260	"	10	10.11

All animals bled by cutting the jugular vein and carotid artery without anaesthesia.

Table XIII.

Guinea-pigs nos.	Weight g.	Diet	No. of days on scorbutic diet	Phosphorus whole blood mg./100 cc.
204, 207	395, 330	Basal and autoclaved milk, 5 cc. decitrated lemon juice	0	34.98
214, 213	340, 310	"	0	43.17
225, 217	335, 275	"	0	35.01
226, 228	285, 325	Basal and autoclaved milk, 10 cc. decitrated lemon juice	0	36.16
233, 236	285, 235	"	0	42.40
234	305	"	0	35.25
205, 218	245, 365	Basal and milk	5	39.62
211, 215	340, 260	"	5	36.64
227, 229	260, 375	"	5	34.10
230, 232	305, 335	"	8	42.81
242, 243	255, 265	"	8	37.91
209, 212	405, 300	"	10	38.39
220, 221	280, 365	"	10	42.10
239, 237	375, 305	"	10	36.46
241	315	"	10	43.65

All animals killed by cutting the jugular vein and carotid artery without anaesthesia.

CONCLUSION.

This investigation reveals no significant change in calcium and phosphorus metabolism in the early stages of scurvy in guinea-pigs. As in the case of nitrogen [Shipp and Zilva, 1928], the balance becomes negative only when the disease has reached the stage at which there is a general disturbance in the physiological functions of the organism, a change which must be regarded as secondary in character and not specific for the disease. The only investigators who have published a complete balance sheet for protected and scorbutic guinea-pigs are Baumann and Howard [1917], who found a negative phosphorus and calcium balance in adult guinea-pigs (400–500 g.) kept on a scorbutic diet of oats and water for 14 days. On examining their figures, however, it becomes evident that this negative balance was not due to the scorbutic nature of the diet but to the low intake of the elements under discussion, since their animals consumed only 5 mg. of calcium and 30–40 mg. of phosphorus daily and lost 32 % of their body weight in the 14 days on the scorbutic diet. This loss is rather high even for guinea-pigs kept in metabolism cages, and was probably due to an insufficient consumption of food in general.

It is also evident from our investigation that the calcium and phosphorus content of the blood of normal guinea-pigs varies considerably, a fact previously recorded by Edelstein and Schmal [1926] for serum and by Teich [1929] for whole blood of these animals. Only very considerable variations in the blood of animals on a scorbutic diet can therefore be accepted as significant. Like Edelstein and Schmal we observed no such changes. In only one experiment is there any change which appears to be associated with the change of diet (Table X: lower calcium and higher phosphorus in the scorbutic than in the normal period) and in this case it is the figures for the normal animals that are outside the usual range. Euler and Myrbäck [1925] noted a fall in the phosphorus content of the blood of scorbutic animals and suggested that this change might be utilised in the early diagnosis of scurvy in these animals. This conclusion was arrived at by Euler and Myrbäck from the examination of the blood of only two normal and three scorbutic guinea-pigs and it is probable that the difference was due to individual variation and not to the deficient diet.

SUMMARY.

There is no deviation from the normal in the absorption or retention of calcium or of phosphorus before or during the development of scurvy in guinea-pigs. Disturbances in the calcium and phosphorus balances are noted only in the last stages of the experiment when all the functions of the animal organism become deranged by the disease.

The calcium and phosphorus content of the blood of protected and scorbutic guinea-pigs varies within wide limits and no difference can be established in this respect between the two groups.

REFERENCES.

- Baumann and Howard (1917). *Amer. J. Med. Sci.* **153**, 650.
Briggs (1922). *J. Biol. Chem.* **53**, 13.
Edelstein and Schmal (1926). *Z. Kinderheilk.* **41**, 30.
Euler and Myrbäck (1925). *Z. physiol. Chem.* **148**, 180.
Kramer and Tisdall (1921). *J. Biol. Chem.* **47**, 497.
Martland and Robison (1924). *Biochem. J.* **18**, 765.
Shipp and Zilva (1928). *Biochem. J.* **22**, 1449.
Teich (1929). *Compt. Rend. Soc. Biol.* **102**, 151.
Zilva and Wells (1919). *Proc. Roy. Soc. Lond. B* **90**, 565.

LXVIII. A NOTE ON THE REPRECIPITATION OF THE ANTISCORBUTIC FACTOR FROM DECITRATED LEMON JUICE.

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IN a recent paper [Zilva, 1930] it was pointed out that when the active precipitate obtained by precipitation with lead acetate at p_H 7.2 from decitrated lemon juice, previously cleared with neutral lead acetate, was dissolved in acetic acid and the reaction brought up with ammonia to p_H 7, no precipitate was formed at that hydrogen ion concentration. Only when the p_H was raised to 8 a precipitate began to appear and the maximum precipitation was obtained between p_H 8 and 9 only. This fraction was found to be totally inactive in doses equivalent to 5 cc. of the original juice. The observation was not in accord with that made by Grettie and King [1929] and Sipple and King [1930] (the latter cleared their decitrated juice in the first place with alcohol) who obtained a second precipitate at p_H 7.2-7.4 which, they claimed, showed very little loss in activity after the reprecipitation.

In dissolving the first precipitate in the above experiment an excess of 10 % acetic acid, namely a volume equal to that of the original juice from which the precipitate was obtained, was used and this yielded a very clear solution. It has been since found that if less acetic acid is used the p_H at which the second precipitate begins to appear is considerably lowered. Thus, with a certain juice it was possible, by using the least quantity of 10 % acetic acid necessary to dissolve the precipitate (about 10 cc. per 50 cc. of the original juice), to lower the p_H at which the precipitate first appears to 6.2-6.9 (average 6.4) instead of 8 previously observed, and consequently a considerable precipitate was obtained when the p_H was further raised to 7.4. The first precipitate began to appear at p_H 5.5-6.0 (average 5.7). On testing the second precipitate for its antiscorbatic potency, it was found that an equivalent of 5 cc. of the original decitrated juice gave very nearly the same degree of protection as an equivalent of 1.5 cc. of the first precipitate; in other words, a loss of rather less than 66 % was incurred in the reprecipitation. In estimating the dry matter in 7 samples of the first and second precipitates, decomposed with $MgSO_4$, during the progress of the biological test, it was found that the loss of solids varied from day to day but that the average amount

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of inactive material removed by the second precipitation amounted to only 33 %, a lower figure than that of the loss incurred in antiscorbutic activity. No purification was therefore effected in this case.

It must be further pointed out that the reducing capacity for phenolindophenol of the first precipitate of this batch averaged 11 cc. of 0.02 % phenolindophenol per equivalent of 5 cc. of the original juice, the highest figure so far obtained by the author. In the second precipitate there was a loss of 40-50 % of this reducing capacity. With the average preparation, however, such a loss would have brought the reduction down to a level which experience has shown to be characteristic of inactive fractions, and a risk is thus run of totally inactivating the fraction by a second precipitation, even when it is brought down at about p_H 6.4. This point will be dealt with in detail in a subsequent publication.

REFERENCES.

- Grettie and King (1929). *J. Biol. Chem.* **84**, 771.
Sipple and King (1930). *J. Amer. Chem. Soc.* **52**, 420.
Zilva (1930). *Biochem. J.* **24**, 1687.

LXIX. THE EFFECT OF DESICCATION UPON THE NUTRITIVE PROPERTIES OF EGG-WHITE. II.

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(Received March 2nd, 1931.)

IN a previous paper [Boas, 1927] it was shown that dried egg-white, when used as the sole source of protein in a diet for young rats, produces a train of characteristic symptoms—notably dermatitis, baldness and spastic gait—terminating in death in from 4 to 6 weeks. It was shown further that this syndrome can be completely prevented if certain substances such as yeast, potato, arrowroot, commercial caseinogen, fresh egg-white and others are added to the diet, and the presence of an unknown protective dietary factor X in these substances was postulated. The symptoms were produced only when the egg-white was dried in the undenatured state, but the results were the same whether dehydration was carried out by evaporation in air at 37°, *in vacuo* in the presence of H_2SO_4 at room temperature, or by treatment with alcohol. The two following alternative hypotheses were advanced as possible explanations of the phenomena:

(a) that dried egg-white is lacking in some essential dietary factor, which is supplied by protective factor X;

(b) that a toxic substance is formed in dried egg-white as a result of desiccation, and that this is neutralised in some way by protective factor X.

The investigation has since been carried a stage further and as there is at present no prospect of continuing it, it seems best to publish the results so far obtained although the work is still incomplete. There are now other workers in the field and it is hoped that the experiments described in this paper may be of assistance to them.

Findlay and Stern [1929], using the same diet as the author, obtained similar results and confirmed the protective power of raw potato, yeast, raw egg-white, egg-yolk and cow's milk. They found raw liver to have a high potency. They suggest that the condition produced in the rats fed upon the dried egg-white diet is analogous to "pink disease" (Swift's disease) in children, the clinical picture of nervous and cutaneous symptoms being similar. An extensive histological study of the rats showed that the most important changes were in the skin, where there was active keratinisation,

and in the spinal cord, where a diffuse infiltration of small round cells into both grey and white matter was revealed. A mild case of Swift's disease examined by them showed a similar infiltration into the spinal cord. Since Findlay and Stern were able to produce the characteristic symptoms in newborn and suckling rats by feeding the mother during pregnancy and lactation on the dried egg-white diet, they favour the hypothesis that the dried egg-white is deficient in some dietary essential, but they admit that the theory that a toxic substance is involved is not excluded by this evidence.

Parsons [1931] has also produced the dried egg-white syndrome in rats, which for the most part received diets containing as much as 66 % of protein in the form of dried egg-white, raw or cooked. In her description of the symptoms Parsons mentions in addition a scaly condition of the tails which the present author has not observed. Parsons found that dried beef-liver, if constituting 20 % of the diet, protected the rats but that 10 % did not and that 20 % of dried yeast was also ineffective.

The present paper is mainly concerned with a series of tests, some of a curative and some of a preventive nature, in which the effects of using some of the different constituents of egg-white, fresh or dried, as the source of protein were investigated. Such tests were expected to produce evidence which was in favour of or could be set against one or other of the alternative hypotheses described above.

DESCRIPTION OF DIETS AND MATERIALS USED.

Table I gives full details of the constitution of the various diets used in these experiments. Each rat received in addition to the diet 3 to 5 drops of cod-liver oil daily. All the egg-white diets were dissolved in water and coagulated by heat before incorporation in the diet.

Table I. *Details of the diets used.*

	DW diet	DG diet	Diets containing separate egg-white proteins							Casein- ogen diet	Liver diet	Steak diet	
			(1 a)	(1 b)	2	3	4	5	6				7
Dried egg-white	100	100	—	—	—	—	—	—	—	50	50	50	50
Pure ovalbumin solution	—	—	100 (dry)	—	—	—	—	—	—	—	—	—	—
Dried pure ovalbumin	—	—	—	100	—	—	—	—	—	—	—	—	—
Dried ovoglobulin*	—	—	—	—	200	—	—	—	16	—	—	—	—
Dried egg-white less globulins	—	—	—	—	—	100	—	—	—	—	—	—	—
Dried albumins and ovomucoid	—	—	—	—	—	—	100	—	91	50	—	—	—
Albumins and ovomucoid from dried egg-white	—	—	—	—	—	—	—	100	—	—	—	—	—
Fresh raw ox-liver	—	—	—	—	—	—	—	—	—	—	250	—	—
Fresh raw beef steak	—	—	—	—	—	—	—	—	—	—	—	250	—
Purified caseinogen	—	—	—	—	—	—	—	—	—	50	—	—	—
Wheat starch	250	—	250	250	250	250	250	250	250	250	250	250	250
Glucose	—	250	—	—	—	—	250 or 250	250	—	—	—	—	—
Hardened cotton seed oil	75	75	75	75	75	75	75	75	75	75	75	75	75
Marmite	25	25	25	25	25	25	25	25	25	25	25	25	25
Salt mixtures†	25	25	25	25	25	25	25	25	25	25	25	25	25
Distilled water	300	300	—	300	300	300	300	300	300	300	300	150	150

* This was 50 % NaCl.

† McCollum, Simmonds and Pitz [1917].

The materials used were prepared as follows.

Dried egg-white. See Boas [1927].

Egg-white fractions. (1 *a*) *Pure ovalbumin.* Ovalbumin crystals were prepared from fresh egg-white by the method of Hopkins and Pinkus [1898], dissolved in water and dialysed against tap-water until free from sulphate, a little toluene being added to prevent decomposition.

(1 *b*) *Pure ovalbumin, dried.* Fraction (1 *a*) was dried at 37°.

(2) *Ovoglobulin, dried.* To raw fresh egg-white an equal quantity of a saturated solution of ammonium sulphate was added and the precipitate filtered off. This was treated with a dilute solution of sodium chloride in order to dissolve the ovoglobulin. Most of the ovomucin remains undissolved [Hektoen and Cole, 1928] and can be filtered off. The ovoglobulin was then treated again with a saturated solution of ammonium sulphate and the precipitate further purified by redissolving it in water and precipitating a third time. It was then dissolved, dialysed as above, taken up with a dilute solution of sodium chloride and dried as usual. This preparation contains a little ovalbumin as well as the ovoglobulin.

(3) *Filtrate after removal of the globulins, dried.* Method (*a*). Raw fresh egg-white from which the globulins had been removed by half-saturation with ammonium sulphate was dialysed as above and dried in the usual way.

Method (*b*). To raw fresh egg-white four volumes of water were added, the whole well mixed and made neutral to litmus with dilute acetic acid. The precipitate of globulins was filtered off and the filtrate dried.

(4) *Albumin and ovomucoid fraction from fresh egg-white, dried.* Fresh raw egg-white, from which the globulins had been removed by half-saturation with ammonium sulphate, was treated with excess of solid ammonium sulphate until no further precipitate could be produced and the filtrate was protein-free. The precipitate was filtered off, dissolved in water, reprecipitated and again filtered off. After dissolving in water it was dialysed and dried.

(5) *Albumin and ovomucoid fraction from dried egg-white, dried.* 200 g. of dried egg-white were dissolved in 1600 cc. of water. From this solution the albumin and ovomucoid fraction was prepared as under (4).

Purified caseinogen. This was prepared by the method described by Chick and Roscoe [1928], to whom I am indebted for the sample used here.

Yeast extract. Pressed washed yeast was heated for 5 hours at 120° and then stirred into a large volume of boiling dilute acetic acid (0.01 %) and boiled for 5 minutes. It was then filtered and the filtrate concentrated so that 1 cc. was equivalent to 0.7 g. of the original dry yeast.

Yeast residue. The residue from the yeast extract was subjected to two further similar extractions and then dried at 37°. 1 g. of the residue was equivalent to 2.2 g. of the original dry yeast.

EXPERIMENTS ON THE PURIFIED PROTEINS OF EGG-WHITE.

Crystalline ovalbumin.

Assuming that crude raw egg-white contains some essential accessory substance X which is destroyed during drying (hypothesis (a) above) one might expect that a pure crystalline constituent protein such as ovalbumin would be freed from this factor. The use of pure ovalbumin as the sole protein of the diet would in that case be followed by the development of the same symptoms as are produced by the use of dried egg-white. Crystalline ovalbumin, prepared as described above, was used as the sole source of protein in the diet of two rats (see Table II), which had developed the usual symptoms as a result of receiving the dried egg-white diet (DW) for 8 weeks. Their condition immediately improved and rapid growth was resumed. It was, however, possible that the essential factor might still remain adherent to the albumin even after crystallisation. The solution of pure ovalbumin was therefore dried at 37°. Curative experiments on 3 rats (see Table II) proved it to have suffered no change in nutritive property.

Table II. *Results obtained when rats which had developed the symptoms as a result of receiving the dried egg-white (DW) diet were given instead diets containing different egg-white fractions as the source of protein.*

Litter No.	Rat No.	Length of time on DW previously (days)	Condition of rat when diet changed		New diet No.*	Result of change of diet		
			Symptoms	Weight		Symptoms	Weight g.	
78	657 ♀	58	Severe	Falling	(1 a)	Healing	Gain of 27 in 16 days	
78	658 ♂	58	"	"	(1 a)	"	" 24 "	16 "
81	686 ♂	Steak diet 51 DW 8	Marked	"	(1 b)	"	" 32 "	11 "
82	697 ♂	21	Appearing	Rising	(1 b)	"	" 67 "	18 "
82	699 ♀	21	"	"	No change	Worse	" 9 "	18 "
84	715 ♂	33	Severe	Falling	(1 b)	Healing	" 37 "	18 "
84	716 ♂	42	"	"	4	"	" 18 "	14 "
84	717 ♀	42	"	"	4	"	" 22 "	18 "
84	718 ♀	42	Marked	"	4	"	" 29 "	18 "
84	714 ♂	42	Severe	Stationary	No change	Worse	Loss of 6 "	9 "
84	719 ♂	42	"	"	"	"	" 4 "	12 "
84	720 ♀	42	"	"	"	"	" 7 "	12 "
86	731 ♀	36	Marked	"	6	Healing	Gain of 26 "	12 "
86	735 ♀	36	Severe	"	6	"	" 12 "	12 "
86	732 ♀	36	Marked	Falling	6	"	" 31 "	12 "
86	734 ♂	36	Severe	"	6	"	" 28 "	12 "
86	736 ♂	36	Marked	"	No change.	Worse	Loss of 8 "	12 "
86	737 ♂	36	Severe	Stationary	3	Died in 7 days		
86	739 ♂	36	"	Falling	3	Worse	Loss of 5 in 14 days	
86	738 ♂	36	"	Stationary	4	Healing	Gain of 29 "	16 "
89	761 ♀	35	Marked	"	2	"	" 48 "	21 "

* See Table I.

These results are opposed to the theory that the symptoms caused by the ingestion of dried egg-white as the sole protein are caused by a deficiency of some dietary essential. One is, therefore, forced to conclude that when egg-white is dried some toxic substance is formed (hypothesis (b)).

The source of the toxic substance.

In the hope of determining from which of the constituents of egg-white the toxic substance is derived some of the other proteins contained in egg-white were examined separately. According to Hektoen and Cole [1928] there are five different proteins in egg-white, ovoglobulin and ovomucin together constituting the fraction precipitable by half-saturation with ammonium sulphate, ovalbumin and conalbumin both precipitated by saturation with ammonium sulphate, the first crystallisable and the second non-crystallisable and ovomucoid. The albumins constitute about 80 % of the total protein, the globulins 7 % and the ovomucoid 10 %.

Use was made of this classification of Hektoen and Cole in preparing the egg-white fractions described above. From these egg-white diets (2), (3), (4) and (6) in Table I were made up and given to rats which had developed the usual symptoms as the result of receiving the DW diet for from 21 to 42 days.

The results of the change of protein are shown in Table II. It will be seen that diets (2), (4) and (6) were harmless, their replacement of the DW diet being followed by healing and resumption of growth. Diet (3), however, containing the whole egg-white dried after removal of the globulin fraction, appeared to be toxic. To confirm this result, 3 rats of one litter were fed from weaning on diet (3), 5 rats from the same litter receiving the DW or DG diets. The remaining 3 rats in the litter received diet (5), containing as the sole protein the albumins and ovomucoid fraction prepared from dried egg-white. At the end of 6 weeks it was found that all the rats with the exception of those fed on diet (5) had developed the usual symptoms, thus confirming the toxic nature of egg-white dried after removal of the globulin fraction. This experiment demonstrated also the important fact that although dried egg-white is toxic, it is possible to prepare from it a non-toxic fraction. This provides further convincing proof that dried egg-white is not lacking in some unknown essential. The growth curves of the rats in this experiment are shown in Fig. 1.

These results show that neither ovoglobulin, conalbumin, ovalbumin nor ovomucoid produces the toxic substance when it is dried. Since the toxic substance is produced when the albumins, the ovomucoid and the protein-free mother-liquor are dried together, ovomucin can also be exculpated. It follows, therefore, that the toxic substance is derived, either from some constituent of the mother-liquor or alternatively from one of the albumins or the ovomucoid when these are dried in the presence of the mother-liquor. Against the latter hypothesis must be set the harmless nature of the albumins and ovomucoid fraction when separated from the rest of the toxic dried egg-white.

One is forced to conclude, therefore, that during desiccation of egg-white a toxic substance is formed from some non-protein constituent and that this substance remains present in the mother-liquor after removal of the proteins by precipitation with ammonium sulphate. This hypothesis cannot be regarded as fully established until it has been shown that the addition of the

dried mother-liquor to an otherwise harmless diet results in the production of the characteristic syndrome, an experiment which has not as yet been carried out.

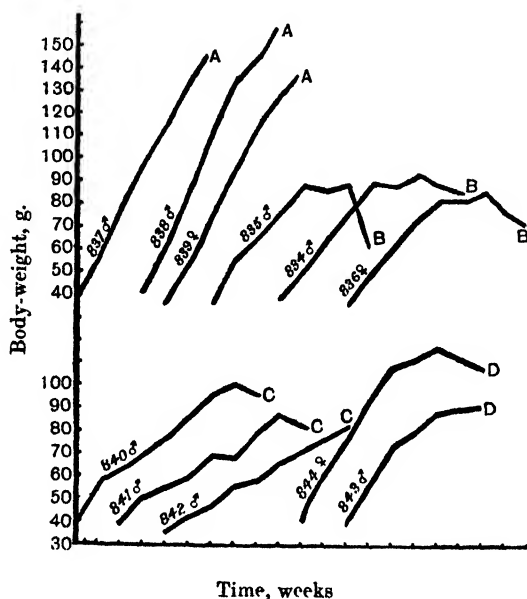


Fig. 1. Growth curves of rats fed from weaning on diets containing as the source of protein:

- A. Albumin and ovomucoid fraction from dried egg-white.
- B. Egg-white dried after removal of the globulin fraction.
- C. Dried egg-white.

In A, B and C the carbohydrate was glucose, in D wheat starch.
All rats were from the same litter.

THE PROTECTIVE FACTOR X.

Solubility. It was shown previously [Boas, 1927] that the protective factor X appeared to be insoluble in water or alcohol. Further evidence on this point has been obtained.

(1) The factor is not removed from caseinogen by repeated washing with dilute acetic acid followed by extraction with hot alcohol. Rats reared from weaning on the caseinogen diet described in Table I, in which the protein consisted of dried egg-white and purified caseinogen in equal quantities, grew well and appeared in perfect health. (It was shown before [Boas, 1927] that a reduction of the dried egg-white in the diet to one-half the usual amount without the addition of any other substance did not decrease the severity of the symptoms produced.)

(2) Only a small amount of the factor is extracted from fresh washed yeast by boiling in dilute acetic acid (0.01 %). A daily dose of yeast extract equivalent to 1.4 g. of dried yeast did little to prevent the syndrome in rats fed upon the DW diet, whereas a dose of the yeast residue equal to 1.3 g. of dried yeast gave good protection.

(3) Fresh egg-white possesses protective power. Complete protection resulted when fresh egg-white was ingested in a proportion to dried egg-white of one to one, but when the ratio was one to three, only partial protection was afforded. It has now been found that when a precipitate of albumins and ovomucoid is formed by saturation with ammonium sulphate of fresh egg-white, from which the globulins have been removed, the protective substances are also carried down. This fraction can neutralise the toxic material in dried egg-white if it is given to rats in the proportion of one part of the fraction to one part of dried egg-white.

The experiments briefly described above are shown in full detail in Table III.

Table III. *Details of experiments showing the presence of protective factor X in certain substances.*

Litter No.	Rat No.	Diet*	Length of exp. (days)	Gain in body-weight g.	Condition of rat at close of exp.
83	705 ♀	DW	41	63	Marked symptoms
83	712 ♂		41	79	"
83	707 ♀		41	67	Good condition
83	708 ♀	Caseinogen	41	79	"
83	709 ♂		41	111	"
83	710 ♂		41	97	"
82	695 ♂	DW	35	—	Dead
82	699 ♀		42	48	Marked symptoms
82	700 ♀		42	42	"
82	702 ♀	DW + daily dose of 2.0 cc. yeast extract (last 3 weeks)	42	66	Moderate symptoms
82	703 ♀		42	69	"
82	696 ♂		42	42	"
82	694 ♂	DW + daily dose of 0.6 g. yeast residue (last 3 weeks)	42	85	Good condition
82	704 ♀		42	72	"
90	772 ♀	DW	47	44	Moderate symptoms
90	773 ♀	Egg-white fraction, diet 7	47	88	Good condition
90	771 ♂		47	85	"
81	683 ♂	DW	42	59	Severe symptoms
81	684 ♂		42	27	"
81	690 ♀		42	25	Marked symptoms
81	685 ♂	Steak diet	42	113	"
81	686 ♂		42	91	"
81	687 ♂		42	89	"
81	691 ♀		42	77	"
81	688 ♂		23	107	Excellent condition
81	689 ♂	Liver diet	23	64	"
81	692 ♀		23	62	"
81	693 ♀		23	71	"

* See Table I.

Distribution in nature. The paucity of the protective factor X in lean meat has been confirmed. Rats reared on a diet (see Table I) in which the protein consisted of 50 g. of dried egg-white and 250 g. of raw lean beef steak (68 g. dry weight) showed a condition little superior to that of the controls receiving DW diet. On the other hand, raw liver is a rich source, for 250 g. (83 g. dry weight) of raw ox-liver proved amply sufficient to counteract the effects of 50 g. of dried egg-white. Parsons [1931] found that the amount of dried beef-

liver needed to neutralise 66 g. of dried egg-white was between 10 and 20 g. and that 20 g. of dried yeast were inadequate. On the other hand, I have found that a rat ingesting from 1 to 2 g. of dried egg-white daily was kept in good health by a daily dose of 0.4 g. of dried yeast. Findlay and Stern [1929] have also confirmed the high protective potency of dried yeast.

REFECTION.

Some interesting light has been thrown on the problem by the discovery that rats showing characteristic symptoms when fed upon dried egg-white sometimes exhibit spontaneous cures. At the same time they produce bulky faeces containing undigested starch. These effects appear to be of a similar nature to the spontaneous cures occasionally observed in the case of rats reared upon a diet devoid of water-soluble vitamins [Fridericia *et al.* 1927; Roscoe, 1927]. In this condition, called refection by Fridericia, the rats pass bulky faeces white in colour with a large content of undigested starch and as long as such faeces are produced the rat is able to grow and enjoy perfect health in spite of the absence of water-soluble B vitamins from the diet. Refection can be transmitted by feeding the faeces of a relected rat and Fridericia *et al.* have postulated the existence of a virus of refection, cultivated in the intestine of the affected rat and passing out in the faeces.

In the spontaneous cures observed in rats receiving the dried egg-white diet the condition could also be passed from one rat to another by ingestion of the faeces. Furthermore, rats showing characteristic symptoms on the dried egg-white diet exhibited cures after receiving doses of the faeces from relected rats (see Fig. 2). It appears, therefore, that the agency in both cases of refection may be identical and this suggests two possibilities. If, as Fridericia suggests, refection is due to the growth in the gut of an organism which synthesises the members of the vitamin B complex, then this organism may also be supposed to synthesise the protective factor X. This is not improbable for yeast has been found to be a rich source of all these factors. On the other hand, the spontaneous cures of the rats fed on the dried egg-white diet may be simply due to some union of the toxic substance with the undigested starch thus preventing absorption of the former.

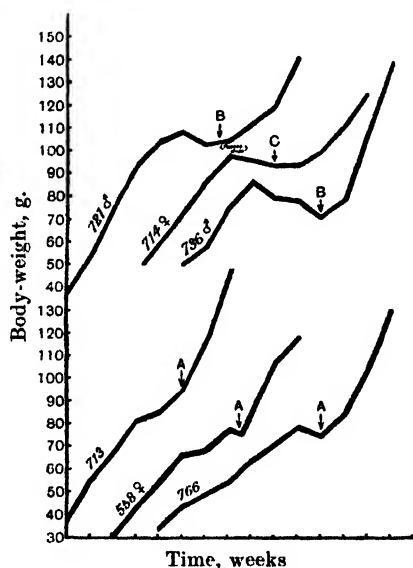


Fig. 2. Growth curves of rats showing:
 At A. Spontaneous cures.
 At B. Cures induced by a daily dose for 7 days of 0.7 g. of the faeces of a relected rat.
 At C. Cure induced by a daily dose for 12 days of 0.5 g. of the faeces of rat 713, showing a spontaneous cure.
 All rats were receiving the DW diet throughout.

DISCUSSION.

The weight of the evidence offered above is certainly on the side of the hypothesis that dried egg-white contains some toxic substance created during the process of drying. It is for the most part opposed to the theory that the nutritive disorder is due to a deficiency. These facts afford little support to Findlay and Stern's proposed identification of the egg-white syndrome with Swift's disease. Their case is based on pathological and clinical findings and until evidence of a therapeutic or dietetic nature is obtained must be regarded as unproved.

The protein-free mother-liquor has been suggested as the source of the toxic substance, but this deduction is based on a process of exclusion and much further work is needed before it can be regarded as established.

It has already been pointed out [Boas, 1927] that the protective factor X presents in its distribution in nature striking analogies with the water-soluble B vitamins, in particular with vitamin B₂. Its presence, however, in purified caseinogen which, so far as is at present known, contains none of the members of the vitamin B complex, and its absence from marmite clearly prove that it is not identical with any of these factors. It seems highly probable that it will ultimately prove to be a closely allied substance.

The conception of a toxic substance in the diet, neutralised by a specific accessory factor, is not a new one in dietetics. Mellanby [1926] has demonstrated a toxamin in oatmeal the action of which is antagonised by vitamin D, and there is considerable evidence that the harmful effects of an excess of certain proteins in the diet can be counteracted by a factor probably identical with one of the constituents of the water-soluble B complex [Hartwell, 1922, 1924, 1925; Reader and Drummond, 1926; Hassan and Drummond, 1927; Parsons, 1931].

SUMMARY.

1. Pure ovalbumin, prepared by crystallisation from crude egg-white, forms a satisfactory protein for young rats. The same is true of ovoglobulin and of the total albumin and ovomucoid fraction.
2. Desiccation does not alter the nutritive properties of these materials as is the case with crude egg-white.
3. It therefore appears probable that the alteration in nutritive value observed when egg-white is dried is due not to the loss of some essential factor but to the formation of a toxic substance.
4. Evidence is adduced suggesting that the toxic substance is formed from some non-protein constituent of the egg-white.
5. It is possible to prepare from dried egg-white a protein fraction uncontaminated by this toxic substance.
6. The protective factor X which is capable of neutralising the toxic substance in dried egg-white is present in raw liver, in a dried preparation of

the total albumin and ovomucoid fraction of egg-white, but not in raw beef steak.

7. It is only partially extracted from yeast by boiling dilute acetic acid.

8. It is not removed from caseinogen by washing with dilute acetic acid and extraction with alcohol.

9. Rats suffering from the effects of the dried egg-white can become spontaneously refected in a similar manner to that observed by Fridericia and his colleagues and by Roscoe in the case of rats fed upon diets devoid of water-soluble B vitamins.

10. This condition can be transmitted from one rat to another by ingestion of the faeces.

11. Refection can be induced in rats, receiving the dried egg-white diet, by feeding the bulky white faeces of refected rats on diets deprived of B vitamins. This suggests that the agency is the same in both cases.

I should like to record my thanks to Sir Charles Martin and to Dr H. Chick for their advice and help throughout the course of the investigation.

The above work, publication of which has been delayed until now, was carried out for the most part during the tenure of a Beit Memorial Fellowship.

REFERENCES.

- Boas (1927). *Biochem. J.* **21**, 712.
 Chick and Roscoe (1928). *Biochem. J.* **22**, 790.
 Findlay and Stern (1929). *Arch. Dis. Childhood*, **4**, 104.
 Fridericia, Freudenthal, Gudjonsson, Johansen and Schoubye (1927). *J. Hyg.* **27**, 70.
 Hartwell (1922). *Biochem. J.* **16**, 825.
 — (1924). *Biochem. J.* **18**, 785.
 — (1925). *Biochem. J.* **19**, 1075.
 Hassan and Drummond (1927). *Biochem. J.* **21**, 653.
 Hektoen and Cole (1928). *J. Infect. Dis.* **42**, 1.
 Hopkins and Pinkus (1898). *J. Physiol.* **23**, 130.
 McCollum, Simmonds and Pitz (1917). *J. Biol. Chem.* **29**, 521.
 Mellanby (1926). *Med. Res. Council Special Rep. Series*, No. 93.
 Parsons (1931). *J. Biol. Chem.* **90**, 351.
 Reader and Drummond (1926). *Biochem. J.* **20**, 1256.
 Roscoe (1927). *J. Hyg.* **27**, 103.

LXX. THE NATURE OF THE LIPOID MATTER EXTRACTED FROM GREEN LEAVES (SPINACH AND CABBAGE).

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The unsaponifiable fraction from the spinach leaf.

CLENSHAW and SMEDLEY-MACLEAN [1929] examined the unsaponifiable matter of the lipid matter of spinach leaves and isolated from it (1) carotene, (2) a saturated hydrocarbon melting at $68-68.5^{\circ}$ which they identified as hentriacontane $C_{31}H_{64}$, and (3) a sterol which forms less than 5 % of the total unsaponifiable matter. The same authors also examined the leaves of green cabbage and showed that they contained a hydrocarbon, which they did not completely characterise, similar to that present in spinach. This was accompanied by a substance containing oxygen which did not appear to be represented in spinach and which was reserved for further investigation.

Shortly afterwards Heyl, Wise and Speer [1929] published the results of an examination of spinach fat in which very much larger quantities of material had been worked up than had been used by the previous observers. The conclusions of these authors differed so widely from those of Clenshaw and Smedley-MacLean that it seemed desirable to repeat the investigation of spinach fat and to endeavour to reconcile the discrepancies.

The hydrocarbon isolated by Heyl, Wise and Speer melted at $67.5-68.5^{\circ}$ and contained C, 84.8 %, H, 15 %: to this they ascribed the formula $C_{20}H_{42}$, apparently on the ground of a single molecular weight determination by Rast's method. Since the analyses and melting-points of the hydrocarbons isolated in the two investigations were completely in agreement and corresponded with those of the hydrocarbon $C_{31}H_{64}$, it appeared necessary to carry out a number of determinations of the molecular weight of the hydrocarbon by Rast's method [1922] to see how far they agreed and also to confirm the composition of the hydrocarbon by submitting it to X-ray examination.

In our hands the values obtained for the molecular weight of the hydrocarbon were 409, 432 and 424, while another observer found 405 and 420 for the same material. We can offer no explanation of the value of 285, the single observation recorded by Heyl, Wise and Speer. Our experience indicates that while Rast's method is exceedingly useful in fixing the approximate molecular weight of a hydrocarbon, it does not afford a basis for a decision between

adjacent members of a homologous series when carried out under the ordinary laboratory conditions of determining melting-points.

We are indebted to Dr Piper for carrying out the X-ray examination. He reports that the hydrocarbon gave a very good photograph spacing 41.3 Å units to $\frac{1}{2}$ %. It gave a large number of orders—about ten measurable—and was probably a highly pure specimen of the hydrocarbon containing 31 carbon atoms.

There is no doubt therefore that Clenshaw and Smedley-MacLean were correct in assigning the formula $C_{31}H_{64}$ to the hydrocarbon present in the unsaponifiable matter of spinach. From the same material Heyl, Wise and Speer claim to have isolated two other substances; (1) an alcohol melting at 76–77° to which they ascribed the formula $C_{22}H_{46}O$ as the result of analysis and of a single recorded observation by Rast's method, and (2) a substance melting at 87–88° containing C, 77.7 %, H, 13.7 %, for the molecular weight of which a single determination by Rast's method gave 272. Presumably as the result of an error in calculation the formula $C_{24}H_{50}O_2$ corresponding with a molecular weight of 370 is ascribed to this substance. Having worked up considerably more material than in the earlier investigation of Clenshaw and Smedley-MacLean, we succeeded in isolating a substance containing oxygen, which agreed in melting-point and in composition with the alcohol described by Heyl, Wise and Speer. The complete separation of all traces of hydrocarbon from the spinach alcohol is attended with some difficulty. We found that the alcohol formed an acetate melting at 61.5° and a urethane melting at 64–65° and closely resembled *n*-ceryl alcohol. A specimen of the latter was prepared from Chinese wax and the addition of this alcohol, its acetate and urethane respectively to the similar compounds derived from the spinach alcohol caused no depression in melting-point. The alcohol described by Heyl, Wise and Speer as $C_{22}H_{46}O$ is therefore identical with ceryl alcohol.

Alcohol	C %	H %	Mean mol. wt. (Rast)	M.P.
Ceryl alcohol ($C_{26}H_{54}O$)	81.67	14.13	382	78–79°
Alcohol from Spinach:				
(a) Heyl, Wise and Speer	80.9	14.2	272	76–77°
(b) Collison and Smedley-MacLean	81.32	13.74	353	77–78°
			365	
			421	

In our investigation the total amount of dry spinach leaves worked up was 6.1 kg. whereas the American investigators started with 68 kg. This probably explains why we did not succeed in obtaining any of the oxygen-containing substance melting at 87–88°, 0.2 g. of which was isolated by Heyl, Wise and Speer.

The sterol from spinach was of particular interest. Unfortunately the amount isolated was not sufficient for an extended investigation. Our analyses agreed with those of the above observers and we confirm the formula $C_{27}H_{46}O$. It is dextro-rotatory, melts at 164°, gives a Salkowski reaction with the reddish

colour in the sulphuric layer, and is highly unsaturated giving an iodine value by Hubl's method of 245. It closely resembles in its properties the much lower-melting zymosterol with which it is isomeric.

The fatty acids of the spinach leaf.

These were examined and palmitic and cerotic acids identified, confirming the result of Speer, Wise and Hart [1929]. From 3600 g. dried spinach leaves, 69.12 g. of material soluble in light petroleum were extracted. On saponifying this with a cold alcoholic solution (6 %) of sodium ethoxide, 25.67 g. of unsaponifiable matter were obtained and after acidification and further purification by boiling with charcoal in solution in light petroleum, 10.5 g. of fatty acids were isolated. The acid fraction was separated by means of the solubilities of its lead salts in ether into a liquid and a solid fraction. The latter was separated into two parts which showed the following properties:

	M.P.	Mol. weight (by titration)
Fraction I	74.5–75.5°	406
(Several times recrystallised)	78–78.5°	—
Cerotic acid	78–79°	410
Mixed M.P.	78–79°	—
Fraction II	50°	266
(Several times recrystallised)	59–61°	—
Palmitic acid	62°	256

The molecular weight of the liquid portion of acids was found by titration to be 259 and its iodine value 205.8; on bromination of this portion, oleic and linolenic acids were identified. A doubly unsaturated acid was also present, but the solubility of its tetrabromide in light petroleum was greater than that expected for the bromide of linoleic acid. It is possible therefore that the doubly unsaturated acid present is either an isomeride of linoleic acid or else a lower homologue. The quantity of material at our disposal did not permit us to investigate this further.

The unsaponifiable fraction from the cabbage leaf.

The amount of lipid material extracted by light petroleum from green cabbage leaves which had previously been dried in air at 37° was similar to that which we had obtained from spinach leaves, namely about 2 %, of the weight of dried leaves. Approximately one half of this consists of unsaponifiable matter. Chibnall and Channon [1927, 1] carried out an investigation on the lipid matter of cabbage, prepared in a somewhat different manner. Their method was to mince the cabbage leaves, to add an equal volume of water to the minced leaves and to express the juice, which was then coagulated at 70°. The coagulum was separated by filtration and pressed so that the residue contained about 40 % of water. The residue when treated with ether gave an extract which was dark green in colour and contained chlorophyll and its decomposition products, whereas the extract which we obtained by the

direct treatment of the leaves (dried at 37°) with light petroleum was light yellow and almost free from chlorophyll.

The lipid matter from the petroleum extract was saponified in the cold by means of 6 % alcoholic sodium ethoxide and gave about half its own weight of unsaponifiable matter. The iodine value of the unsaponifiable matter obtained from the green leaves of large summer cabbage was 100 compared with a value of 57.5 for that obtained from the pale yellow heart leaves of the same cabbage. The iodine value of the unsaponifiable matter prepared from a green unheaded winter cabbage was 58.5. The difference in iodine value is probably explained by differences in carotene content [Collison, Hume, Smedley-MacLean and Smith, 1929] for the carotene content of white leaves is much less than that of green leaves and the winter cabbage also proved less effective as starting material for the preparation of carotene than the large-headed summer cabbage. The iodine value of carotene determined by the same method as that used for the unsaponifiable matter of cabbage was 330 and the proportion of carotene present in the unsaponifiable matter must therefore greatly influence the iodine value of the unsaponifiable material.

The unsaponifiable material was treated with hot alcohol: most of the carotene remained in the sticky insoluble residue and from this a specimen melting at 178° was prepared. In the hot alcoholic solution there remained the hydrocarbon and oxygen-containing compound previously noticed by Clenshaw and Smedley-MacLean. A full description of these substances was published by Channon and Chibnall [1929] shortly afterwards and they were identified as *n*-nonacosane, $C_{29}H_{60}$, and *n*-dimyristyl ketone respectively. We found that the best method of separating these substances is by washing the mixture with small quantities of cold light petroleum, in which the hydrocarbon is more readily soluble. When the mixture containing a large proportion of the ketone was converted to the oxime by the method described by Channon and Chibnall and the substance obtained after oximation dissolved in acetic acid, the precipitate which separated when the solution was cooled melted at 55°-60°. On the addition of alcohol to the filtrate, short needles separated melting at 57.5°. When these were mixed with palmitone oxime (m.p. 58-59°) the melting-point of the mixture was depressed by 5°. The oxime of the dimyristyl ketone described by Channon and Chibnall melted however at 50-51°. In another similar preparation of the oxime starting with a different batch of material, the oxime which separated melted at 52-53°. Both specimens, melting respectively at 52-53° and at 57.5°, gave the same results on analysis and from both on treatment with acid the same ketone melting at 80° was recovered. We are now investigating the reason for this difference in melting-point, which we cannot at present explain.

The fatty acids of the cabbage leaf.

From 2800 g. of dry cabbage leaves, 14.3 g. of fatty acids insoluble in water were separated and these were divided by the usual lead salt method into 7.21 g. of liquid and 1.6 g. of solid acids.

The solid acid fraction was separated by repeated crystallisation into two parts melting respectively at 62–64° and 80–80.5°. So little material was available that the definite identification of these fractions presented considerable difficulties. The lower melting fraction appeared to consist chiefly of palmitic acid. The molecular weight determined by titration of the fraction of M.P. 80° was 357, a value intermediate between those required for behenic acid ($C_{22}H_{44}O_2$, M.P. variously given as 77–78°, 80–82°, 84°) and lignoceric acid ($C_{24}H_{48}O_2$, M.P. 80.5°). An ethyl ester was prepared from the small amount of acid available and melted at 51–52°. The ethyl ester of behenic acid melts at 56–57°.

Chibnall and Channon [1927, 2] give the following figures for the identification of the fractions from the 1.45 g. of solid acids isolated by them from cabbage:

		C %	H %	M.W.	M.P.
Fraction (1)	0.38	75.23	12.60	271	54–55°
(2)	0.65	75.18	12.43	262	54–55°
(3)	0.34			266	—

They point out that these figures would be given by a mixture of 30 % of stearic and 70 % of palmitic acid and hence they assume that these acids are present. A mixture of 90 % palmitic acid with 10 % behenic acids would give the following figures:

C %	H %	M.W.
75.19	12.61	264

From the evidence now brought forward it is clear that the evidence for the presence of stearic acid in the cabbage leaf is very doubtful, since in this plant the palmitic acid present is associated with a small proportion of a higher saturated acid, probably behenic.

In view of the recent identification of the liquid acids as oleic, linoleic or its homologue, and linolenic, we did not further investigate the liquid fraction of the acids.

General remarks on the lipid material extracted from green leaves.

From the investigation of the leaves of cabbage and spinach and from the evidence available, chiefly in the work of Power, Tutin and their collaborators, it seems probable that the higher members of the normal paraffin series and their derivatives must play an important part in the metabolism of the plant leaf.

Speaking generally the bulk of the acids of the leaf seem to be the unsaturated acids. Linolenic, linoleic or an isomeride, oleic and an unsaturated acid containing 16 carbon atoms have been recorded. The proportion of solid acids is small and of these palmitic acid seems to be an almost invariable constituent and in a large proportion of instances has been isolated in the pure state. Smaller amounts of one or other of the higher saturated fatty acids appear commonly to be present. Stearic acid is frequently noted though the evidence for its presence often consists of the analysis of a fraction of fatty acids of lower melting-point and slightly higher carbon content than palmitic acid. This mixture is sometimes obtained by recrystallisation and sometimes by saponification of a fraction obtained by distillation of the methyl esters, but in many cases the evidence adduced for the presence of stearic acid is unconvincing. From the evidence in the literature cerotic appears to occur most commonly of the higher fatty acids and several instances are recorded of the occurrence of arachidic, behenic, and melissic acids.

The higher fatty alcohols also occur; ceryl alcohol has been found in spinach leaves in the present investigation, in hops [Power, Tutin and Rogerson, 1913], in *Prunus serotina* [Power and Moore, 1909] and in other plants.

The occurrence of these higher fatty alcohols and acids is of interest in connection with the presence of the higher paraffin hydrocarbons in leaves. Thorpe and Holmes [1901] isolated from tobacco leaves two hydrocarbons melting respectively at 59.3–59.8° and 67.8–68.5°, and identified them as the normal paraffin hydrocarbons containing 27 and 31 carbon atoms. Since that time, the analyses of a large number of leaves, stems and flowers have been made chiefly by Power, Tutin and their collaborators. In a somewhat cursory survey of the literature we have noted the isolation of these hydrocarbons from 39 different plants. From 21 of these hentriacontane ($C_{31}H_{64}$), melting at 68° or in its near neighbourhood, has been separated. This hydrocarbon is the one most often recorded and is presumably derived from palmitic acid, by conversion of the latter to palmitone and by the subsequent reduction of the latter. We can however in the papers we have examined find no record of the presence of palmitone in the same material and we have been unable to find any evidence of the presence of this ketone in the spinach leaf. The presence of pentatriacontane $C_{35}H_{72}$ which bears a similar relation to stearic acid to that existing between hentriacontane and palmitic acid is recorded nine times: heptacosane $C_{27}H_{54}$ presumably derived from myristic acid appears five times. Nonacosane $C_{29}H_{60}$, M.P. 63–64°, was isolated by Pickles [1911] from the leaves and stems of bog myrtle and recently by Channon and Chibnall from cabbage. There also occurs nine times the description of triacontane $C_{30}H_{62}$, a hydrocarbon which alone of those described contains an even number of carbon atoms and which could not therefore be derived by the method postulated from any one single fatty acid. The evidence for the existence of this seems weaker than for that of any of the other hydrocarbons described and it appears not improbable that the so-called triacontane should really be

regarded as nonacosane, since the evidence rests chiefly on the determination of the melting point and this in all cases approximates closely to that of the nonacosane.

	M.P.	Source	
Nonacosane	63–64°	Bog myrtle	Pickles [1911]
	(uncorr. 62.7–62.8°)	Cabbage	Channon and Chibnall [1929]
Triacontane	64–65°	<i>Anthemis nobilis</i>	Power and Browning
		flowers	[1914, 2]
	60–61°	Cotton-root bark	[1914, 1]
		<i>Euphorbia pilulifera</i>	[1913]
	63–65°	<i>Matricaria chamomilla</i>	[1914, 3]
		flowers	
	65.2°	<i>Eriodictyon</i> leaves	Power and Tutin [1907]
	65.5°	<i>Cluytia similis</i>	Tutin and Clewer [1912]
	65.5°	<i>Solanum angustifolium</i>	[1914]
		leaves and flowers	

Since Levene, West and van Scheer [1915] find that the melting-point of synthetic triacontane is 69–70°, the examination of the naturally occurring hydrocarbon which has been identified as triacontane needs revision; there are certainly grounds for believing that it may be identical with nonacosane.

If triacontane may be eliminated, the existence of hydrocarbons containing only odd numbers of carbon atoms agrees with their derivation from the fatty acids containing respectively 14, 15, 16 and 18 carbon atoms. The difficulty of obtaining the solid saturated acids from leaves in sufficient quantity to make a complete identification renders it difficult to say whether the acid with 15 carbon atoms does occur in the plant leaf. Acids with odd numbers of carbon atoms are described, as for instance, that with 21 carbon atoms which is several times recorded [*e.g.* Tutin and Clewer, 1912] and it may well be that α -oxidation of palmitic acid takes place in the plant leaf. The occurrence of dimyristyl ketone in the cabbage leaf together with the hydrocarbon derived from it by reduction is of great interest and suggests that the fatty acid containing 15 carbon atoms must occur at some stage in the metabolism of the leaf.

SUMMARY.

1. The unsaponifiable matter of the spinach leaf contains in addition to carotene and a sterol, $C_{27}H_{46}O$, the hydrocarbon hentriacontane, $C_{31}H_{64}$, the identification of which has been confirmed by X-ray examination. Ceryl alcohol $C_{26}H_{54}O$ is also present. Palmitic, cerotic, oleic, linolenic acids and an isomeride of linoleic acid have been isolated.

2. The identification of nonacosane and *n*-dimyristyl ketone in the unsaponifiable matter of the cabbage leaf [Channon and Chibnall, 1929] is confirmed. The saturated fatty acids are palmitic and a higher fatty acid, probably behenic.

3. The question of the occurrence of the higher paraffin hydrocarbons in the leaf and their probable derivation is discussed.

We desire to acknowledge our gratitude to the Department of Scientific and Industrial Research for a grant which has given us the opportunity to

carry out this work and to express our thanks to Dr Piper for his kindness in carrying out the X-ray examination of the hydrocarbon.

REFERENCES.

- Channon and Chibnall (1929). *Biochem. J.* **23**, 168.
Chibnall and Channon (1927, 1). *Biochem. J.* **21**, 225.
—— — (1927, 2). *Biochem. J.* **21**, 479.
Clenshaw and Smedley-MacLean (1929). *Biochem. J.* **23**, 107.
Collison, Hume, Smedley-MacLean and Smith (1929). *Biochem. J.* **23**, 634.
Heyl, Wise and Speer (1929). *J. Biol. Chem.* **82**, 111.
Levene, West and van Scheer (1915). *J. Biol. Chem.* **20**, 521.
Pickles (1911). *J. Chem. Soc.* **99**, 1764.
Power and Browning (1913). *Pharm. J.* [iv] **36**, 506.
—— — (1914, 1). *Pharm. J.* **93**, 420.
—— — (1914, 2). *J. Chem. Soc.* **105**, 1829.
—— — (1914, 3). *J. Chem. Soc.* **105**, 2280.
—— and Moore (1909). *J. Chem. Soc.* **95**, 243.
—— and Tutin (1907). *J. Chem. Soc.* **91**, 887.
—— Tutin and Rogerson (1913). *J. Chem. Soc.* **103**, 1267.
Rast (1922). *Ber. deutsch. chem. Ges.* **55**, 1051.
Speer, Wise and Hart (1929). *J. Biol. Chem.* **82**, 105.
Thorpe and Holmes (1901). *J. Chem. Soc.* **79**, 982.
Tutin and Clewer (1912). *J. Chem. Soc.* **101**, 2221.
—— — (1914). *J. Chem. Soc.* **105**, 1845.

LXXI. THE CUPROUS DERIVATIVES OF SOME SULPHYDRYL COMPOUNDS.

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METALLIC derivatives of mercaptans have been long known and in the cases of mercury and lead extensively studied. The copper derivatives on the other hand, though they have been prepared on numerous occasions, have received comparatively little attention and have rarely even been analysed. It is one of the purposes of this paper to point out that the formation of a cuprous compound is a general reaction of mercaptans, and that such compounds are frequently very well suited for their isolation or purification.

Precipitates have frequently been obtained on adding a solution of a cupric salt to a mercaptan in neutral or slightly acid solution, but Klason [1877], using thioglycollic acid, first pointed out that it was a cuprous compound that was formed while some of the mercaptan was oxidised to disulphide. Before this Carius [1862] had prepared copper derivatives of mono-, di- and tri-thioglycerol; his analytical figures show that these contain univalent copper.

Suter [1895] and Gabriel [1905] as well as several more recent workers, have prepared mixtures of cuprous cysteine and cuprous chloride by adding copper sulphate solution to a solution of cysteine hydrochloride, while Mörner [1904] and Friedmann [1903] have investigated the cuprous derivatives of α - and β -thiolactic acids. It is probable also that the greyish-black precipitate which Lewis and McGinty [1922] obtained on adding copper sulphate to an extract of the urine of a rabbit which had been fed on phenylcarbamidocysteine was the cuprous compound of phenylcarbamidocysteine.

So far the methods for the preparation of cuprous mercaptides have all involved the conversion of half the mercaptan to the corresponding disulphide, and this renders them unsuitable for preparing pure mercaptans. Hopkins's [1929] striking observation, that cuprous oxide would react with a mercaptan in acid solution to form the cuprous mercaptide, has changed the whole situation, since by this method, with a little care, a mercaptan may be quantitatively precipitated as its cuprous derivative and so obtained pure.

EXPERIMENTAL.

Part I. *The preparation of some cysteine and cystine derivatives.*

Acetylcysteine. Diacetylcysteine was first made by Inouye [1929] by the hydrolysis of various diacetylcysteine esters. Working at 37°, and adding the

theoretical amount of sodium hydroxide gradually so that the p_{H} never exceeds 9.5 (0.2 g. of glycine may conveniently be added to buffer the fluid at this p_{H}) I find that the hydrolysis of 5 g. of the methyl ester of diacetylcystine dissolved in 200 cc. of water takes 6 hours. The propyl ester, the use of which Inouye recommends, is much less soluble and the saponification of 5 g. suspended in 600 cc. of water requires 9 hours at 60°.

These methods are laborious and there is great risk of racemisation during the hydrolysis. A simpler method was therefore sought. Neither acetyl chloride nor acetic anhydride will react with cystine in pyridine suspension, but the latter, as Nicolet [1930] and Du Vigneaud and Hollander [1930] have also found, reacts smoothly in alkaline solution.

10 g. of cystine are dissolved in 50 cc. of 10 % sodium hydroxide, 16 cc. of acetic anhydride are added and the mixture is kept cool and stirred until homogeneous. The excess of acetic acid and acetic anhydride is removed by distillation at 40° *in vacuo* till a viscous gum remains. This is dissolved in 300 cc. of 4 % (by volume) sulphuric acid and reduced with 5 g. of zinc dust; the mixture is stirred occasionally and filtered after about 30 minutes. An aqueous suspension of cuprous oxide is cautiously added to the filtrate, care being taken that, after each addition, the precipitate has lost its initial pink colour before more cuprous oxide is added. The end-point is easily found by centrifuging a sample and adding cuprous oxide to the centrifugate, but, should it be overshoot and the cuprous compound of acetylcysteine redissolve, the latter may be quantitatively re-precipitated by aeration. This phenomenon has already been described in the case of glutathione [Pirie, 1930].

The colourless cuprous compound is washed by repeatedly stirring up the precipitate in distilled water and centrifuging. After 3–4 washings the centrifugate remains cloudy even after 15 minutes at 3500 R.P.M. It is then sulphate-free and contains some dissolved cuprous acetylcysteine. On suspending the thoroughly washed solid in water and decomposing with H_2S (by the closed vessel method) a suspension of cuprous sulphide resembling strong coffee is obtained. This is perfectly transparent when diluted and the sulphide cannot be removed by centrifuging or filtering through kieselguhr. The solution is evaporated to complete dryness *in vacuo* at 45° and the residue is boiled up with glacial acetic acid. The cuprous sulphide may now be removed by filtration and the filtrate, after concentration by vacuum distillation, is evaporated to crystallisation in a desiccator over soda-lime. Whetstone-shaped crystals of acetylcysteine form readily on scratching the concentrated acetic acid solution with a glass rod.

The yield is variable, depending on the completeness of the precipitation with cuprous oxide but is generally about 6.5 g. M.P. (uncorr.) 107°–109°. (Found: N, 8.52 %; S, 19.9 %; Calc.: N, 8.59 %; S, 19.63 %.)

Chloroacetylcysteine. Fischer and Suzuki [1904] isolated dichloroacetylcystine by extracting with ether the solution obtained when chloroacetyl chloride is added to an alkaline solution of cystine. This method gives a pure

unhydrated product, but it is troublesome when used on a large scale. The hydrate, slightly contaminated with coloured products, will however crystallise from the acidified reaction mixture on standing for a day in the ice-chest.

To 22.3 g. of cystine, dissolved in 188 cc. of *N* NaOH, 16.7 cc. *i.e.* 25 g. of chloroacetyl chloride and 275 cc. of *N* NaOH are added concurrently in the course of an hour, the temperature being kept below 5° throughout. After standing at room temperature for 2 hours 34 cc. of concentrated hydrochloric acid are added and the mixture is filtered after being well shaken. It is put in the ice-chest and next day the hydrated dichloroacetylcystine is filtered off and washed with cold water. The yield at this stage is 27 g. and a further 3–5 g. of a less pure material may be obtained by distilling the filtrate and washings *in vacuo* until an oil separates, and cooling.

4 g. of hydrated dichloroacetylcystine and 1 g. of zinc dust are shaken at room temperature for an hour with 120 cc. of 2.5 % H_2SO_4 . The dichloroacetylcystine and most of the zinc dissolve. Some H_2S is formed. If the reaction mixture is heated, or if a large excess of zinc is used, there is more of this destruction and some free hydrochloric acid, which interferes with the subsequent precipitation by cuprous oxide, is also formed. Dewar and Gamgee [1871] observed the production of H_2S during the reduction of cystine by tin, and some H_2S is almost always formed during the reduction of a disulphide by zinc even in the cold, but it is more marked in the case of dichloroacetylcystine than in that of any other compound that I have used.

The solution is filtered and, if necessary, air is blown through it to remove the H_2S ; after warming to about 40° cuprous oxide suspension is added very gradually. When about half of the cuprous oxide has been added the action proceeds more rapidly and the mixture frequently sets to a perfectly rigid jelly which breaks up into a curd on stirring. Cuprous chloroacetylcysteine is soluble in presence of excess of cuprous oxide but is re-precipitated on aeration. Owing to the relative insolubility of chloroacetylcysteine in water it is unnecessary to wash the cuprous compound very thoroughly and a few washings with water on a Büchner funnel are adequate.

As in the case of acetylcysteine a colloidal solution of cuprous sulphide is formed if cuprous chloroacetylcysteine is decomposed with H_2S in aqueous suspension. When suspended in glacial acetic acid, however, especially in the presence of the electrolytes which were not removed by washing, the Cu_2S separates after a few hours as a precipitate. It is filtered and the filtrate, after concentration by distillation *in vacuo*, is evaporated in a vacuum desiccator over soda-lime. The crystals are recrystallised from water. Chloroacetylcysteine generally forms irregular angular masses but occasionally small regular octohedra are obtained. Yield 2–2.5 g. m.p. 126°. (Found: N, 6.95 %; S, 16.67 %; Cl, 17.34 %; Calc.: N, 7.09 %; S, 16.2 %; Cl, 17.97 %.)

Glycylcysteine. 4 g. of hydrated dichloroacetylcystine are put in a strong 100 cc. flask and 20 cc. of ammonia solution (sp. gr. 0.880) are added. Since the neutralisation of the acid by ammonia evolves considerable heat it is best

to add 5 cc. of ammonia first with cooling and then, when the solid has again reached room temperature, the remainder. The flask is tightly stoppered and kept in an incubator at 37° for 4 days. So far the method is simply a modification of that of Abderhalden and Spinner [1919] for the preparation of diglycylcystine.

As much of the excess ammonia as possible is removed by distilling the brownish-coloured solution *in vacuo* till it forms a gum adhering to the sides of the flask, this is dissolved in 20 cc. of 2 % sulphuric acid. To the acid solution 2.9 g. of finely powdered silver sulphate are added and the mixture is left, with occasional stirring, for some hours. It is important to precipitate all the chlorine at this stage. After filtering, 40 cc. of 2 % sulphuric acid are added to the filtrate, followed, after removing any excess of silver with H_2S , by 1–2 g. of zinc dust. Reduction is complete in about 20 minutes. The excess of zinc is filtered off and the filtrate heated to about 80° before any cuprous oxide is added. Precipitation will occur at a lower temperature if all the hydrochloric acid has not been removed or if a considerable amount of cuprous glycylcystine has already been formed. If however cuprous oxide is added to a cold acid solution of glycylcystine most of it is converted to cupric oxide and copper. As in the case of the two compounds already described an excess of cuprous oxide will cause re-solution of the already precipitated cuprous compound. On aeration it is re-precipitated as a bright yellow powder which, apart from its colour, is identical with the flocculent white mass which is formed at first.

The removal of the last traces of sulphuric acid from this substance is difficult, and as the properties of the resulting glycylcystine show, I have probably never accomplished it quite completely. It is advisable to use a filtration method of washing, similar to that which has been described already [Pirie, 1930], rather than to wash by centrifuging. The cuprous glycylcystine is somewhat unstable in the absence of acid and gradually becomes greenish on exposed surfaces when the washing has been carried nearly to completion. It is suspended in water and decomposed with H_2S . Decomposition is very rapid and after a few hours the solution may be filtered from cuprous sulphide. It is advisable to keep the volume of water used to suspend the cuprous compound as small as possible and to evaporate the filtrate (still containing some H_2S) rapidly in a vacuum desiccator over sulphuric acid and caustic soda. Residual sulphuric acid keeps the p_{H} of the resulting gum down to about 4 and makes crystallisation difficult. It will always crystallise however when seeded.¹ Owing to the excessive solubility of glycylcystine in water the mother-liquor in contact with it is very viscous and filtration is slow. A

¹ After several early failures crystals were obtained by adding pyridine to the hydrochloric acid-containing gum which resulted when the treatment with silver sulphate was omitted. On washing with alcohol fairly pure glycylcystine was obtained, but it is difficult to make the removal of pyridine hydrochloride by alcohol under these conditions complete. Since then I have experienced no difficulty with crystallisation.

further quantity of less pure material may be obtained by adding alcohol to the filtrate. As usual the yield is variable but 5–6 g. may be expected.

Glycylcysteine has no definite melting-point. It sinters at 130° and then gradually swells to a semi-solid foam which melts again at 177° and goes brown. (Found: N, 15.28 %; S, 18.08 %; Calc.: N, 15.73 %; S, 17.97 %.)

Methyl ester of acetylcysteine. The disulphide is prepared by a method based on that of Inouye [1929]. A suspension of 6.8 g. of cystine dimethyl ester hydrochloride in 50 cc. of dry pyridine is cooled to 0° and 4 cc. (excess) of acetyl chloride added gradually. Considerable heat is developed and the ester hydrochloride dissolves when about half the acetyl chloride has been added; crystals separate on adding the remainder. After standing at room temperature for 3 hours it is filtered through a small Hirsch funnel. Great care must be exercised here since the white crystals which remain on the funnel are apt to turn brown and melt, and occasionally ignite, on exposure to air. As soon as the fluid has been sucked through the funnel is removed from the flask and washed out. I have attempted to avoid this by adding the theoretical amount of acetyl chloride, but this results in a very much diminished yield. The filtrate is evaporated in a vacuum desiccator over sulphuric acid till crystallisation commences. On mixing with its own volume of water a mass of crystals is obtained and filtered off. The filtrate is brought to about p_H 6 with 10 % sodium hydroxide; on removing most of the liberated pyridine in a vacuum desiccator further crystallisation takes place. The crystals are washed with cold water.

5.9 g. of colourless crystals, m.p. 128–9°, are obtained. Unlike Inouye, I find that the dimethyl ester of diacetylcysteine is perfectly stable in air and not at all hygroscopic. (Found: N, 7.95 %; S, 18.19 %; Calc.: N, 7.95 %; S, 18.18 %.)

0.380 g. was made up to 10 cc. with water and the rotation measured in a 19.95 cm. polarimeter tube.

Using the two mercury lines the rotations were -7.35° and -6.53° these values gave $[\alpha]_{577}^{17} = -96.94^\circ$ and $[\alpha]_{546}^{17} = -86.13^\circ$. The latter figure is in good agreement with the value (-85.59°) given by Voss, Guttmann and Klemm [1930].

Zinc dust is added to 2 g. of diacetylcysteine dimethyl ester dissolved in 150 cc. of 2 % sulphuric acid in the cold. The solution is filtered and treated with cuprous oxide at about 30° in the usual way. After washing, the cuprous acetylcysteine ester is suspended in glacial acetic acid and decomposed with H_2S . The cuprous sulphide remains colloidal even on boiling so the suspension is evaporated to dryness *in vacuo* and boiled with benzene. The benzene solution is filtered and evaporated in a desiccator over paraffin after most of the benzene has been removed on a water-bath. The residue may be recrystallised from water containing a little acetic acid and melts at 80°. (Found: N, 7.94 %; S, 18.35 %; Calc.: N, 7.91 %; S, 18.08 %).

Diacetylcysteine dipropyl ester. Propyl alcohol is dried by distilling from lime and 6 g. of cystine are suspended in a litre. Hydrogen chloride is passed

into the mixture, boiling under a reflux condenser, for 2 hours. It is filtered hot and evaporated to small bulk by vacuum distillation. 6 g. (M.P. 178–9°) of cystine dipropyl ester hydrochloride separate out on cooling with ice. A further crop of crystals separates on adding ether.

7 g. of ester hydrochloride are acetylated in 100 cc. of pyridine with 4.2 cc. of acetyl chloride. After lying overnight in the ice-chest the solution is filtered and the filtrate distilled *in vacuo* to remove most of the pyridine. On adding ether 5.9 g. of diacetylcystine dipropyl ester separate. On recrystallising from water masses of fine needles are formed which are not hygroscopic and melt at 124–5°. (Inouye gave 175° and 117–8° as the melting-points of cystine dipropyl ester hydrochloride and diacetylcystine dipropyl ester respectively.) (Found: N, 6.51 %; S, 15.55 %; Calc.: N, 6.86 %; S, 15.69 %.)

Dicinnamylcystine dimethyl ester. 12 g. of cinnamyl chloride are added in the course of 5 minutes to a cooled suspension of 10.5 g. of cystine dimethyl ester hydrochloride in 200 cc. of dry pyridine. The mixture is then left for 8 hours at room temperature. The pyridine is distilled off *in vacuo* till crystals separate. These are filtered off, after standing overnight in the ice-chest, and the filtrate is poured into water. Both the crystals and the solid which separates when the pyridine filtrate is poured into water are recrystallised from alcohol. The product crystallises in very pale yellow leaflets which melt at 161–2°. (Found: N, 5.06 %; S, 12.21 %; Calc.: N, 5.30 %; S, 12.12 %.)

0.3024 g. of dicinnamylcystine dimethyl ester are dissolved in acetic acid and made up to 10 cc. At 17° the rotations for the mercury lines are -1.21° and -0.82° in a 2 dm. tube, *i.e.* $[\alpha]_{377}^{17} = -20^\circ$ and $[\alpha]_{346}^{17} = -13.6^\circ$.

Oxidised glutathione. Hopkins [1929] observed that when glutathione was oxidised by aeration at p_H 7.6 in the presence of a trace of iron, the product isolated, after adding sulphuric acid to remove the baryta used to adjust the p_H , by evaporation followed by treatment with alcohol, had a low nitrogen and sulphur content. Very slow oxidation in the absence of iron was found to give similar results so other oxidising agents were tried. Hydrogen peroxide proved to be very much the most suitable.

A 10–20 % solution of glutathione is used and exactly the theoretical amount of 30 % hydrogen peroxide is added. Copper catalyses the oxidation of the —SH group at this p_H (2.8) and in the presence of 1 mg. of copper the oxidation of 10 g. of reduced glutathione is complete in 3 hours. So long as an excess of hydrogen peroxide is not added no side reactions occur and 1 mol. of H_2O_2 will cause the complete disappearance of the nitroprusside reaction from a solution containing 2 mols. of glutathione. Glutathione prepared from its cuprous compound may contain less than 1 part of copper per million. It is generally necessary therefore to add a trace of copper sulphate or oxide. When oxidation is complete the solution is cooled to 0°, put in a vacuum desiccator, and exhausted rapidly so that it freezes. The vacuum is maintained and in the course of a day all the ice evaporates leaving the oxidised glutathione as a white feathery non-hygroscopic mass easily soluble in water. It has not

yet been crystallised. Oxidised glutathione prepared in this way gives analytical figures for N and S identical with those of the reduced glutathione used in its preparation.

Mason [1931, 2] in a very recent paper has pointed out that oxidised glutathione holds alcohol tenaciously. It is obvious therefore that, apart from its very great convenience, the method of preparation of oxidised glutathione just described has no merit except the avoidance of alcohol. The presence of alcohol explains Pirie and Pinhey's [1929] observation that the oxidised glutathione used contained 17 % of an impurity which had no titratable groups.

Part II. *Preparation and properties of the cuprous derivatives of some thiol compounds.*

Cysteine. Although mixtures of cuprous cysteine and cuprous chloride have been prepared by several different workers, no one appears to have prepared pure cuprous cysteine.

l-Cystine (*dl*-cystine behaves in a substantially similar way though the cuprous compound of *dl*-cystine is rather more soluble in weak acid than that of *d*-cystine) is dissolved in 2 % sulphuric acid and some zinc dust is added. The solution is boiled and filtered. Both titration with iodine and polarimetric observation indicate that 97–100 % of the cystine has been reduced.

Cuprous cysteine is not readily precipitated from this solution in the cold. A reddish or grey precipitate is obtained and a considerable amount of copper sulphate is formed. Precipitation occurs smoothly in the cold if a trace of hydrochloric acid or a chloride is present, but in the absence of chloride it will take place at the boiling point. Being fairly soluble in boiling dilute sulphuric acid, cuprous cysteine may remain dissolved, but it precipitates on cooling. It is soluble in 5 % sulphuric acid even in the cold, but shows no sign of being soluble in presence of excess of cuprous oxide.

For analysis it is washed a little on the centrifuge and then transferred to a small Jena glass filter of the type used in micro-analysis. Washing is continued, with suction, until the wash-water is sulphate-free or until the cuprous compound begins to turn green. The latter happens first in the case of cysteine. The filter is put in a desiccator over P_2O_5 at room temperature and exhausted to 1 mm. A hard cake is obtained in this way which is much less readily oxidised by air than the loose mass which results if the precipitate in the centrifuge tube is dried.

A sample, containing about 6 mg. of copper, is transferred to a weighed 100 cc. Kjeldahl flask, with all but 8 cm. of its neck cut off, and incinerated with 0.5 cc. of sulphuric acid and some potassium sulphate. When cold, saturated sodium carbonate solution is added till the solution is alkaline. It is then acidified with acetic acid and, after the addition of some solid potassium iodide, titrated with *N*/100 thiosulphate using starch as indicator. If the incineration has been thorough the end-point is perfectly sharp. The

ordinary Pregl method of analysis for copper has been found not to work satisfactorily with many of these compounds.

21.1 mg. of the dried material required 11.1 cc. of *N*/98 thiosulphate whence, Cu, 34.2 %; Calc. Cu, 34.6 %.

Like other compounds of this type, cuprous cysteine is soluble in dilute HCl and in alkalis, it is also soluble in unusually dilute sulphuric acid, for most cuprous compounds are not appreciably soluble in sulphuric acid weaker than 20 %.

Acetylcysteine and chloroacetylcysteine. The cuprous derivatives of these two substances are similar in their general properties. Both are soluble in presence of excess of cuprous oxide and in moderately concentrated (10–20 %) sulphuric acid. They are also soluble in water in the absence of acid; thus on washing a sample by centrifuging, the p_H of each successive centrifugate is higher till a p_H of about 3.6 is reached. At this point the compound goes into solution and the wash-water is found to be sulphate-free. A saturated solution of cuprous acetylcysteine at p_H 3.6 contains 44 mg. of copper per 100 cc. On adding a little sulphuric acid, mixing rapidly and allowing the mixture to stand, a rigid jelly is obtained in about 15 minutes. The concentration of cuprous acetylcysteine in this jelly is 0.15 % so that it shows a gel-forming capacity of the same order as that of dibenzoylcysteine [Wolf and Rideal, 1922]. Cuprous chloroacetylcysteine is less soluble in water (it forms a solution of about 0.07 %) and, although it also will form remarkably rigid jellies, gel-formation is not so reliable as with cuprous acetylcysteine.

When re-precipitated from solution cuprous acetylcysteine does not dry readily and tends to blacken. As a result the analytical figures are generally low: *e.g.* directly precipitated; different preparations contained 27.9, 27.5 and 28.1 % Cu: re-precipitated; different samples contained 25.1, 22.1, 25.0, 24.8 and 26 % Cu: Calc. for $C_5H_8O_3NSCu$ 28.2 % Cu.

Cuprous chloroacetylcysteine also is not very stable on drying and always gives rather low analytical figures. (Found: 23.1 % Cu; Calc.: 24.5 % Cu.)

The p_H of an aqueous solution of cuprous acetylcysteine gradually rises on exposure to air and may reach 7. At this stage nothing is precipitated by acid and the fluid will dissolve further quantities of solid with consequent fall in p_H . It is probable that this rise in p_H is due to the oxidation of cuprous copper to the cupric form. This then combines with the carboxyl instead of with the sulphhydryl group. A titration curve indicated that, while there was a group with a p_K in the neighbourhood of 4, there was no other titratable group with a p_K less than 12. This suggests that, as would be expected, the copper in cuprous acetylcysteine is linked to the sulphur atom.

If either compound is dissolved in 2 % sulphuric acid and put in a narrow-mouthed vessel, with a stream of hydrogen passing through to prevent oxidation, the initially formed cuprous compound is completely redissolved on the gradual addition of two atomic proportions of copper, in the form of cuprous oxide suspension of known strength. If too little cuprous oxide is

added, traces of the normal cuprous compound remain undissolved, if too much is added metallic copper deposits. The solution formed is yellow or brown. For every 10 cc. of solution 100 cc. of 97 % alcohol are put into a Büchner flask and the solution is filtered directly into it through a previously moistened paper. A yellowish gelatinous precipitate is formed but it is too unstable to give consistent analytical figures; probably, by analogy with glutathione, it is a complex of two molecules of cuprous acetylcysteine and one of cuprous sulphate.

Glycylcysteine. Like cysteine this substance forms a cuprous compound in the cold in the presence of a chloride, but in the absence of chloride it is necessary to heat to 80° before adding the cuprous oxide. It resembles cysteine also in forming a cuprous compound from which the sulphuric acid is not readily removed by washing. It is perfectly stable when dried. (Found: 27.36 %, 26.77 % and 26.6 % Cu; Calc.: 26.44 % Cu.)

Cuprous glycylcysteine reacts readily with more cuprous oxide when it is suspended in 6 % sulphuric acid in the absence of air. The product obtained by filtering the solution into alcohol and then washing the precipitate with alcohol till it starts to turn green is stable when dry. (Found: Cu, 30.7 %; SO₄, 23.06 %)

Calc. for (C₅H₉O₃N₂SCu)₂Cu₂SO₄ Cu 36.1 %, SO₄, 13.63 %.

Calc. for C₅H₉O₃N₂SCu.CuSO₄ Cu 31.7 %, SO₄, 24.0 %.)

It is thus apparent that the analytical figures are in much better agreement with a formula in which there is one atom of cupric copper than with one in which both are cuprous. Nevertheless, the fact that re-solution of cuprous glycylcysteine occurs on the addition of one atomic proportion of cuprous copper in absence of air and without any free copper being formed, taken in conjunction with the fact that cuprous glycylcysteine is re-formed on aeration (analysis of the yellow material shows that it contains 26.7 % Cu), seems to me to indicate that both atoms of copper are in the cuprous state. Sulphuric acid is a probable contaminant in view of the necessarily incomplete washing, although 9.43 % is undoubtedly more than would have been anticipated.

Methyl and propyl esters of acetylcysteine. Both react normally in acid solution and give cuprous derivatives which dissolve on the addition of another atomic proportion of cuprous copper. So far I have been unable to isolate these double copper compounds since they are not only soluble in alcohol but are also very readily autoxidisable.

Propyl acetylcysteine was prepared from the disulphide by suspending it in 2 % sulphuric acid at about 50° and adding zinc dust. The disulphide is rather difficult to wet but it is reduced in about half an hour. The cuprous derivatives of both these compounds are insoluble in water but tend to remain in suspension in water when they have been washed free from acid. The cuprous sulphide suspensions formed on decomposing either of these with

H_2S are remarkably stable and, if free from electrolytes, will withstand boiling with alcohol or glacial acetic acid. Acetylcysteine propyl ester has not been obtained crystalline.

Both cuprous compounds are stable on drying:

$\text{CH}_3\text{CONHCH}(\text{COOCH}_3)\text{CH}_2\text{SCu}$ Found; 26.00 % Cu; Calc. 26.55 % Cu.

$\text{CH}_3\text{CONHCH}(\text{COOC}_3\text{H}_7)\text{CH}_2\text{SCu}$ Found; 23.2 % Cu; Calc. 23.77 % Cu.

Glutathione. Cuprous glutathione has been more extensively studied than the other cuprous compounds. Hopkins [1929] observed that it redissolved in presence of an excess of cuprous oxide. For some reason it will not re-precipitate on aeration when an excess of cuprous oxide has been added to a solution recovered from a mercury precipitate from a boiled yeast extract, whereas, as I have already pointed out [Pirie, 1930], it may be re-precipitated from a yeast filtrate prepared directly. This is due, in part at any rate, to the formation of interfering substances (cysteinylglycine?) on boiling and to their concentration in the filtrate from mercuric sulphate. A certain amount of re-precipitation is obtained if such a solution is largely diluted with water and aerated.

On dissolving glutathione in dilute sulphuric acid and adding cuprous oxide in a current of hydrogen to exclude air, maximum precipitation is obtained after the addition of one atomic proportion of copper. The addition of a second atom of copper results in complete re-solution, but a third may be added before any turbidity due to metallic copper appears. A white precipitate is obtained if the filtrate from this mixture is allowed to run into alcohol. On washing this precipitate it turns slightly green but seems to be stable.

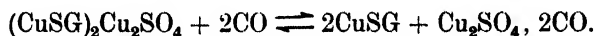
The analytical figures obtained depend on the thoroughness with which it is washed with alcohol. On very thorough washing: Cu, 25.5 % and 25.45 %; SO_4 , 14.2 % and 14.9 %; Calc. for $[(\text{CuSG})_2\text{Cu}_2\text{SO}_4]_2\text{SO}_4$:¹ Cu, 25.1 % and SO_4 , 14.25 %. With less thorough washing the figures are 26.44 % and 16.13 % respectively.

In this case it is obvious that all the extra copper cannot be in the cupric state since there is not enough sulphate present. The figures show further that there are only two atoms of copper present for each atom of sulphur whereas three may be added without any deposition of copper occurring. It is noteworthy that products having the same analytical figures are obtained irrespective of whether two or three atomic proportions of copper have been used to dissolve the glutathione. In the latter case there is some copper in solution in the alcohol. Probably therefore a compound of the type $\text{CuSG} \cdot \text{Cu}_2\text{SO}_4$ is present in aqueous solution, but on pouring the solution into alcohol it is broken down. In view of the extreme difficulty experienced in washing all the sulphuric acid out of certain cuprous compounds, e.g. that of glycylcysteine,

¹ GSH is reduced glutathione.

it seems probable that the substance actually precipitated by alcohol is $(\text{CuSG})_2\text{Cu}_2\text{SO}_4$ and that the extra sulphate is adventitious.

The behaviour of the soluble copper compound towards carbon monoxide is interesting. Joannis [1903] showed that sulphuric acid saturated with carbon monoxide would dissolve finely divided copper and isolated Cu_2SO_4 , 2CO , H_2O . If, without admitting any air, the hydrogen which was used to exclude air during the solution of cuprous glutathione in an excess of copper oxide, is replaced by a stream of carbon monoxide, precipitation of cuprous glutathione occurs. Precipitation is nearly complete in 3 hours. At this stage, if the CO is displaced by a current of hydrogen, there is complete solution of the precipitate. On passing in more CO precipitation occurs again, and so on. The behaviour of CO is thus entirely different from that of oxygen and seems to involve the reaction:



If the suspension obtained by saturating with carbon monoxide is centrifuged, with a stopper in the centrifuge tube to prevent too much loss of CO , and the centrifugate is exhausted until it boils and then left for a day with occasional re-exhaustion, a precipitate of copper is formed. The fluid, which initially liberated very little iodine from potassium iodide, now liberates a large amount. This behaviour is what would be expected if the centrifugate contained the carbon monoxide complex of cuprous sulphate.

Glycylcysteine anhydride. Oxidised glutathione, prepared by the method already described, is boiled in water to form the diketopiperazine by the method of Hopkins [1929]. The resulting disulphide is reduced with zinc. Towards cuprous oxide the reduced product behaves exactly like glycylcysteine except that it is unnecessary to heat the solution to more than 30° before adding the cuprous oxide. The cuprous compound turns greenish when washed and is not very stable. (Found: Cu , 28.48 %; Calc.: Cu , 28.57 %.)

In acid solution the cuprous compound is soluble in presence of an excess of cuprous oxide but it does not appear to stabilise a whole molecule of cuprous sulphate as does glutathione although it might have been expected to do so since it also has two peptide linkages. The gelatinous product obtained on pouring this solution into alcohol is not sufficiently stable to give consistent analyses.

Methyl ester of cinnamylcysteine. Since the dimethyl ester of dicinnamylcystine is insoluble in water it is reduced with zinc in glacial acetic acid containing a few drops of concentrated sulphuric acid per 100 cc. The solution is boiled, filtered and then, when again boiling, treated with cuprous oxide in aqueous suspension. A yellowish curd separates which is washed with acetic acid and then with water till sulphate-free. (Found: Cu , 18.9 %; Calc. for $\text{C}_{13}\text{H}_{14}\text{O}_3\text{NSCu}$: Cu , 19.4 %.)

A suspension of cuprous sulphide formed on decomposing this, suspended in glacial acetic acid, with H_2S but it filtered after being boiled. A white curd

separated on adding water to the concentrated acetic acid solution. (Found: N, 4.81 %; S, 11.50 %; Calc. for $C_{13}H_{15}O_3NS$: N, 5.3 %; S, 12.12 %.)

It gives a nitroprusside reaction and, when oxidised, decolorises an acetic acid solution of bromine. On pouring this solution into water a curd containing 13 % of bromine is obtained. It is therefore improbable that the reduction has saturated the double bond.

Benzoylcysteine. This compound has not been obtained pure. Dibenzoylcystine is shaken with zinc in a mixture of equal volumes of 2 % sulphuric acid and alcohol. It is advisable to add a trace of copper sulphate to catalyse the action. If necessary the mixture is warmed but as a rule all the dibenzoylcystine dissolves easily. After filtering from undissolved zinc and removing the alcohol by distillation *in vacuo*, an oil separates which does not solidify on cooling. This is washed by boiling up again with water and dried in a vacuum. It remains viscous.

Equal volumes of 3 % sulphuric acid and alcohol are used to dissolve this gum and a trace of sodium chloride is added. On adding cuprous oxide to the hot fluid a fawn-coloured precipitate is formed. There is no sign of this being soluble in presence of excess of cuprous oxide. For analysis it is suspended in water in an atmosphere of hydrogen, and dilute sodium hydroxide solution is added. The suspension is centrifuged and the centrifugate poured into dilute sulphuric acid. There is no sign of gel formation and the white precipitate is washed and dried as usual. (Found: Cu, 21.4 %; Calc. for $C_{10}H_{10}O_3NSCu$: Cu, 22.1 %.)

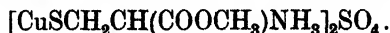
When suspended in alcohol and decomposed with H_2S the cuprous sulphide is not colloidal though it is when suspended in acetic acid. Benzene precipitates benzoylcysteine from its concentrated alcoholic solution as a stringy mass and I have not yet obtained it in satisfactory form.

Cysteinylglycine. Mason [1931, 1] showed that cysteinylglycine did not form a cuprous compound insoluble in sulphuric acid. The compound, prepared by the method of Kendall, Mason and McKenzie [1930], dissolves cuprous oxide in acid solution. There is no precipitation on adding sodium hydroxide till the p_H is about 4, but on evaporating this brown fluid *in vacuo*, a grey precipitate is formed. On exposing this to air it immediately turns chocolate in colour. This abnormal behaviour of cysteinylglycine is difficult to understand, but further investigation of it is in progress. Preliminary work shows that the benzoyl derivative of cysteinylglycine forms a cuprous compound of the usual character.

Cysteine methyl ester. The amount of saturated silver sulphate solution necessary to combine with all the hydrochloric acid is added to some cystine dimethyl ester hydrochloride together with the same volume of 2 % sulphuric acid. The mixture is centrifuged and some zinc is added, after about 10 minutes it is filtered. To the filtrate, cuprous oxide suspension is added, air being excluded by a current of hydrogen. No insoluble cuprous compound is formed, but the copper oxide dissolves with the formation of a perfectly colourless

solution. Two atomic proportions of copper may be added in this way but a third results in a pale green colour and a faint turbidity.

When poured into alcohol a white precipitate forms and the alcohol takes on a bluish tinge. The precipitate rapidly turns grey and does not give consistent analyses. It appears however to have the composition



The alcohol contains more copper than the precipitate. This indicates that, as in the case of glutathione, the more complex copper compound is unstable in alcohol.

DISCUSSION.

Several reasons for the formation of soluble cuprous compounds suggested themselves at the beginning of this work but all have been excluded except one.

A carboxyl group cannot be concerned since the methyl ester of acetylcysteine and glycylcysteine anhydride both dissolve when an excess of cuprous oxide is present.

An amino-group cannot be concerned since acetylcysteine, chloroacetylcysteine, acetylcysteine methyl ester and glycylcysteine anhydride all form soluble compounds.

It is improbable that subsidiary valencies of the sulphur atom are concerned since cysteine and thioglycollic acid do not form soluble compounds. (The cuprous compounds of thiourea and thioglycollic acid have been prepared by several different workers; I find that neither will dissolve on adding an excess of cuprous oxide.)

The peptide linkage remains as a possibility and it seems to be a highly plausible one. Cuprous benzoylcysteine it is true does not dissolve in presence of an excess of cuprous oxide; but benzoylcysteine itself is insoluble in dilute acids unlike the other compounds used. A more serious objection is that none of the sulphur-free peptides which I have tried (*i.e.*, glycylglycine, diglycylglycine and leucylglycylglycine) will stabilise cuprous oxide in dilute sulphuric acid. It seems therefore that, if the theory is correct, the copper mercaptide linkage in the molecule is a necessary precursor of any further action.

The examples given in this paper should serve to show the value of the cuprous compound as a means of separating mercaptans from the other products of a reaction and of getting a pure product without having to recrystallise. Different mercaptans cannot of course be easily separated from each other, though in some cases, *e.g.* a mixture of cuprous acetylcysteine and cuprous glycylcysteine, it is possible to concentrate the two compounds in different fractions.

With the exception of some purines, mercaptans seem to be the only compounds which form cuprous derivatives insoluble in acid solution. A purine-like body, containing 30–36 % of nitrogen, has in fact been isolated from a yeast extract, prepared in the way already described [Pirie, 1930], by adding a large amount of cuprous oxide to the fluid after as much glutathione as possible

has been removed. A yellowish precipitate forms which on washing and decomposing with H_2S gives a white solid soluble in acids, alkalis and H_2S solution but insoluble in water. It has not been further investigated.

In view of the solubility of the cuprous derivatives of cysteine methyl ester and cysteinylglycine and of the difficulty experienced in washing cuprous glycylcysteine or cuprous cysteine free from sulphuric acid, it is advisable to work with acidic rather than with neutral or basic compounds. When dealing with an unknown sulphydryl compound it would therefore be wise to acetylate or benzoylate an amino-group before proceeding to cuprous precipitation.

Attempts to utilise the specificity of this cuprous compound precipitation in a method for the estimation of glutathione have so far been unsuccessful. The most hopeful results were obtained when a tissue extract, warmed to $50-60^\circ$, was titrated with a mixture of copper sulphate and sodium hypophosphite. A precipitate was obtained and, on adding an excess of the reagent, it redissolved giving a fairly sharp end point. The figures obtained were however always very high and were much less reliable than those obtained by iodimetric methods.

The specific rotations of the sulphydryl compounds prepared have not been given in the text since they are unreliable, partly because of the possibility that some of the disulphide is present and partly because the observed angle is so small. The following values for $[\alpha]_{5461}^{17}$ were observed in acid solution:

Acetylcysteine + 0.42° ; chloroacetylcysteine + 8.7° ; glycylcysteine + 6.9° ; methyl ester of acetylcysteine - 31.0° .

In describing the effect of carbon monoxide on the cuprous sulphate complex of a copper mercaptide only glutathione has been mentioned. All the other sulphydryl compounds which form soluble cuprous derivatives with an excess of cuprous oxide, but insoluble derivatives with one atom of copper, behave in precisely the same way.

SUMMARY.

Acetylcysteine, chloroacetylcysteine, glycylcysteine, the methyl ester of acetylcysteine, and the dimethyl ester of cinnamylcysteine have been prepared and the method of preparation is given in each case.

Improvements are suggested in the preparation of oxidised glutathione, dichloroacetylcystine and the dimethyl and dipropyl esters of diacetylcystine.

Cinnamylcysteine methyl ester, acetylcysteine propyl ester and benzoylcysteine have been prepared in an impure state.

The cuprous derivatives of these compounds have been prepared and complexes of these derivatives with cuprous sulphate have in many cases been isolated and analysed. These complexes are decomposed reversibly by carbon monoxide and irreversibly by oxygen.

The suitability of cuprous derivatives for the isolation and purification of sulphydryl compounds is emphasised.

My thanks are due to Sir F. G. Hopkins for the interest he has shown in this work and to the Master and Fellows of Emmanuel College for a Research Studentship during the tenure of which the work was carried out.

REFERENCES.

- Abderhalden and Spinner (1919). *Z. physiol. Chem.* **106**, 299.
Carius (1862). *Liebig's Ann.* **124**, 238.
Dewar and Gamgee (1871). *J. Anat. Physiol.* **7**, 142.
Du Vigneaud and Hollander (1930). *Proc. Soc. Exp. Biol. Med.* **28**, 46.
Fischer and Suzuki (1904). *Ber. deutsch. chem. Ges.* **37**, 4576.
Friedmann (1903). *Hofmeister's Beiträge*, **3**, 1.
Gabriel (1905). *Ber. deutsch. chem. Ges.* **38**, 639.
Hopkins (1929). *J. Biol. Chem.* **84**, 269.
Inouye (1929). *Bull. Inst. Phys. Chem. Res. (Tokyo)*, **8**, 647.
Joannis (1903). *Compt. Rend. Acad. Sci.* **136**, 615.
Kendall, Mason and McKenzie (1930). *J. Biol. Chem.* **88**, 409.
Klason (1877). *Liebig's Ann.* **187**, 120.
Lewis and McGinty (1922). *J. Biol. Chem.* **53**, 349.
Mason (1931, 1). *J. Biol. Chem.* **90**, 25.
—— (1931, 2). *J. Biol. Chem.* **90**, 409.
Mörner (1904). *Z. physiol. Chem.* **42**, 351.
Nicolet (1930). *J. Biol. Chem.* **88**, 395.
Pirie (1930). *Biochem. J.* **24**, 53.
—— and Pinhey (1929). *J. Biol. Chem.* **84**, 321.
Suter (1895). *Z. physiol. Chem.* **20**, 564.
Voss, Guttman and Klemm (1930). *Biochem. Z.* **220**, 340.
Wolf and Rideal (1922). *Biochem. J.* **16**, 548.

LXXII. THE ACTION OF DYESTUFFS ON ENZYMES.

I. DYESTUFFS AND OXIDATIONS.

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DURING the past year, a series of investigations has been carried out in this laboratory on the toxic action of dyestuffs on enzymes and on the catalytic activities of tissues. About thirty dyestuffs of various types have so far been examined and it is already clear that not only do the dyestuffs vary very widely amongst themselves in their attack upon enzymes but that the enzymes exhibit a considerable specificity of behaviour towards the dyestuffs. Marston [1923] showed that the proteolytic enzymes are specifically precipitated by dyes of the safranine type, the compounds produced still being proteolytically active. In the investigations to be described, however, the main object has been to determine the amount of poisoning action a dyestuff has upon an enzyme, and how far the union of dyestuff and enzyme is reversible. Whilst this work was in progress, Bamann and Schmeller [1931] published a paper showing that substances of the indicator type, *e.g.* phenolphthalein, bromothymol blue, *etc.*, have a toxic action on lipase and there is little question but that a study of the action of these substances and of dyestuffs, such as those which are discussed in this paper, will lead to valuable information bearing on the constitution of enzymes.

The fact that dyestuffs have specific toxic actions which can be accurately estimated is a very great aid in distinguishing between enzymes which are closely related and between which there appears at present to be no satisfactory method of differentiation. Moreover, as will be shown subsequently, it is possible, by making use of the knowledge that certain dyestuffs have toxic actions upon an enzyme which is normally intracellular, to gain information on the permeability of cells (*e.g.* bacteria) to these dyestuffs.

The enzymes at present under investigation are those concerned with oxidations and reductions and also with hydrolytic changes, *e.g.* fumarase, urease, diastase.

The present communication is concerned with the effects of dyestuffs upon the oxidations of a number of substrates by bacteria (*B. coli*), and by muscle and brain tissues, succinate in particular being investigated.

EXPERIMENTAL.

The method, in brief, was to determine the amount of oxygen consumed in a certain time by a suspension of bacteria, or a quantity of muscle or brain tissue, in presence of a substrate, and to compare this with the oxygen consumed after the bacteria or tissue had been exposed to the action of a dyestuff (usually at a concentration of 1/5000) for 30 minutes at 37°. The consumption of oxygen was measured in the Barcroft differential manometer, all experiments being carried out at 37°.

There were placed in each vessel of the apparatus 1 cc. of a suspension of bacteria, 1 cc. of $M/5$ phosphate buffer, p_H 7.4, and 0.5 cc. of a 1/1000 solution of the dyestuff. The apparatus was shaken at 37° at the usual rate for half an hour, the taps of the vessels being open. After this interval, 0.5 cc. of a solution of the substrate under investigation was placed in the right hand vessel and 0.5 cc. of saline in the left hand vessel. The taps were closed after a few minutes' further shaking to secure temperature equilibrium, and the rate of oxygen uptake was measured. A control experiment, in which water was substituted for the dyestuff, was carried out in every case. The rates of oxidation were approximately linear, and the oxidation was allowed to proceed for 2 hours (or a less period if this proved more convenient). The oxygen uptakes in a series of experiments at the end of this period were measured and the percentage inhibition effected by the dyestuff calculated. To give an example, the oxygen taken up in $1\frac{1}{2}$ hours at 37° by 1 cc. of a suspension of *B. coli*, unexposed to dyestuff, in presence of 0.067 M sodium lactate was 1367 mm.³ The oxygen uptake by the same amount of the suspension of *B. coli* under the same conditions but after exposure to Congo red (1/5000) was 1161 mm.³, and after exposure to ethyl violet (1/5000) 201 mm.³ The percentage inhibition in the first case was 15 and in the second case 85.

The acid substrates were used in the form of their sodium salts, the p_H of solutions being 7.4.

The *B. coli* suspension was prepared by 48 hours' growth in tryptic broth, the organism being centrifuged from the broth and washed twice with normal saline. It was made into a thick suspension in normal saline and stored at 0°.

In the cases of muscle and brain, 0.5 g. of the tissue was used. Muscle, after dissection from the animal, was cooled and minced. Brain was similarly treated, the whole brain being used.

Choice of dyestuffs.

A fairly representative series of dyestuffs has been chosen. This includes 10 members of the triphenylmethane series (malachite green, brilliant green, methyl violet, crystal violet, ethyl violet, gentian violet, acid green, acid fuchsine, water blue, soluble blue), 3 monoazo-dyestuffs (orange G, chrysoidine, and crystal scarlet) and 3 tetrazo-dyestuffs (Congo red, benzopurpurine, and

Bismarck brown). There is 1 diphenylmethane dyestuff, auramine. The pyronine series is represented by pyronine, eosin and erythrosin; the thiazines by methylene blue and toluidine blue; the safranines by safranine and methylene violet; and the eurhodines by neutral red and Janus green. There are also included acriflavine, haematoxylin, and an anthocyanin, cyanin chloride. It is proposed later to add to this series, but it has been felt that there are sufficient at present under investigation to indicate the main features of the action of dyestuffs on enzymes.

Dyestuffs and oxidations by B. coli.

The results of the action of dyestuffs on the oxidations by *B. coli* of glucose, lactate, succinate and formate are shown in Table I, the final concentrations of these substrates in the Barcroft vessels being 0.017 *M*, 0.067 *M*, 0.067 *M*, and 0.05 *M*, respectively.

It will be seen that, under the experimental conditions employed, whereas 15 of the dyestuffs have a relatively large inhibitory action on the oxidation by *B. coli*, the remaining 14 have little or none.

With regard to the toxic dyestuffs it may be imagined, in the first instance, that the toxicity of the dyes to the oxidations of *B. coli* is due primarily to the lethal action of these substances. Previous evidence, however, has shown with certainty that lethal action is not necessarily connected with a poisoning action on the oxidative mechanisms of the cell. For instance, toluene, which has a lethal action on *B. coli*, does not inhibit the oxidative action of this organism on succinate, lactate or formate [Quastel and Wooldridge, 1927]. Again ultra-violet light which induces great mortality among the cells of *B. coli* has little action on the oxidation by this organism of glucose or lactate [Cook and Stephenson, 1928]. It would be unreasonable, therefore, to ascribe the action of the dyestuffs which inhibit the oxidations of *B. coli* to a lethal action of these substances. An analysis of the results shown in Table I bears out this conclusion. Firstly it will be seen that in many cases the oxidation of lactate or glucose is affected to a greater extent than that of succinate. If a lethal action were responsible, such difference in effects would not be anticipated. Secondly, a specificity of behaviour is observable, for whereas one dyestuff will inhibit lactate oxidation much more powerfully than that of succinate, another will inhibit both to the same extent. Such specificity of behaviour would not be expected if the dyestuffs acted solely by a lethal action.

It is more reasonable to suppose that the toxic action of the dyestuffs is due to their combination with certain enzymes involved in the oxidations. It would be expected, if this were true, that the dyestuffs which are toxic would also be capable of staining the cell and this actually is the case. The reverse conclusion, however, *i.e.* that a dyestuff capable of staining the cell is necessarily toxic to oxidations, does not follow; for (*a*) the combination of enzyme

Table I. *Percentage inhibitions by dyestuffs of oxygen uptakes by B. coli in presence of various substrates. $p_H = 7.4$. Temp. 37° .*

* Dyestuff (1/5000)	Nature of dyestuff	Glucose	Lactate	Succinate	Formate
Malachite green	Basic	100	92	72	71
Brilliant green	"	68	66	67	46
Methyl violet 6 B	"	100	88	46	86
Crystal violet	"	97	91	53	92
Ethyl violet	"	82	85	69	84
Gentian violet	"	98	80	36	75
Methylene blue	"	57	33	75	28
Toluidine blue	"	46	38	72	24
Auramine	"	93	87	51	54
Acriflavine	"	100	95	45	58
Neutral red	"	92	72	37	50
Janus green	"	100	69	29	45
Chrysoidine	"	95	34	19	44
Pyronine	"	39	20	6	17
Safranine	"	98	86	22	52
Methylene violet	"	19	5	10	25
Bismarck brown	"	0	4	8	27
Acid fuchsin	Acid	0	0	2	0
Acid green	"	0	17	3	0
Soluble blue	"	0	0	5	9
Water blue	"	0	8	7	0
Orange G	"	0	4	2	14
Crystal scarlet	"	1	0	7	16
Congo red	"	0	15	10	0
Benzopurpurine	"	0	31	22	22
Eosin	"	4	21	2	3
Erythrosin	"	6	18	16	9
Haematoxylin	"	17	8	39	35
Cyanin chloride	"	15	12	14	13

and dyestuff may not be such as to prevent activation of the substrate occurring and (b) the dyestuff may combine with structures in the cell which are not connected with the enzymes under consideration. It may be pointed out, in this connection, that the compounds of safranine and proteolytic enzymes are still proteolytically active and that methylene violet which has but little toxic action on the oxidations of *B. coli* (under the conditions employed, see Table I) will stain a smear of *B. coli* as well as some of the dyestuffs which are powerfully toxic.

The most important fact which emerges from a study of the results embodied in Table I is that all the highly toxic dyestuffs are basic in character, whilst the acid dyestuffs have comparatively little or no action at the concentrations and p_H used. This leads to the conclusion that the dehydrogenases responsible for the activation of glucose, *etc.*, are essentially acidic in character and are able to adsorb or combine with the basic and not with the acidic dyestuffs under the experimental conditions employed.

This conclusion explains the fact that dehydrogenases, in presence of their substrates, do not reduce dyestuffs in the order expected from their oxidation-reduction potentials. It is clear that the rate of reduction is dependent not only on the reduction potential of the dye but on its access to, *i.e.* its adsorption or combination with, the centre responsible for the activation of the substrate. The basic dyestuffs are more accessible, the acidic less

so, and it is thus found that the sulphonated (acidic) dyes are reduced at a much smaller speed than would be anticipated from the magnitude of their reduction potentials [Quastel and Wooldridge, 1927].

It is of considerable interest that Bismarck brown, which is a basic dye, has but little toxic action on the oxidations of *B. coli*. It is therefore apparent that although basicity of a dyestuff plays an important part in determining the combination of dyestuff and dehydrogenase, the basicity is not by any means the only factor involved; the structure of the dyestuff molecule is also concerned. It is presumably this fact which explains the relatively different actions of the dyestuffs on the oxidations of succinate and lactate. The importance of even slight differences in the structures of the dyestuffs is seen in the effects of safranin and methylene violet. The former is highly toxic to the oxidations of *B. coli*, the latter negligibly so.

It is evident from these results that it is of the highest importance, in using dyestuffs, *etc.*, for the measurement of r_H of cells, to have some knowledge of their effects on the oxidative mechanisms of the cells. Entirely fictitious results will be obtained by the use of dyestuffs having selective and toxic actions.

It is of interest that cyanin chloride¹, a natural anthocyanin, has but little action on the oxidative mechanisms of *B. coli*. It would be expected *a priori* that the natural colouring matters would not have a poisoning action on the oxidative mechanism of the cell.

Phosphates and the toxic action of dyestuffs.

The results given above all relate to suspensions of *B. coli* exposed to a 1/5000 aqueous solution of dyestuff in presence of phosphate buffer at p_H 7.4. It is found when using the organism at various concentrations and of varying activity, that (quantitatively) variable results are obtained. This is particularly the case with methylene blue, with which, in examining the effect of the dyestuff on the oxidation of succinate by *B. coli*, an inhibition of 70 % has been sometimes observed and at other times of only 30 %. The reason is doubtless partly connected with the state of the organism, but it is connected to a far greater degree with the nature of the constituents present in the solution in which the organism is suspended.

In the course of an investigation on the action of methylene blue on the rate of succinate oxidation by *B. coli*, it was found that the presence of phosphate had a most marked influence on the toxicity of the dyestuff. To study the rate of oxidation in the absence of phosphate, use was made of veronal buffer, p_H 7.4, made up as suggested by Michaelis [1930]. It was shown in the first place that the replacement of phosphate buffer by veronal buffer of the same p_H (7.4) had little or no effect on the rate of oxidation of succinate by normal *B. coli*. If, however, the *B. coli* were exposed to methylene blue (1/5000) in presence of veronal buffer, p_H 7.4, for 30 minutes at 37°

¹ We are indebted to Miss Rose Scott-Moncrieff for a specimen of this substance.

previous to the addition of the succinate, the oxidation of the latter was inhibited by over 90 %; whereas exposure to the dyestuff under the same conditions but in the presence of phosphate buffer, p_H 7.4, resulted in an inhibition of about 30 %. The following figures illustrate this:

O_2 uptake in $1\frac{1}{2}$ hours in mm.³ at 37° (in presence of 0.067 *M* succinate) by:

1 cc. <i>B. coli</i> in presence of phosphate buffer, p_H 7.4	665
1 cc. <i>B. coli</i> in presence of veronal buffer, p_H 7.4	625
1 cc. <i>B. coli</i> , previously treated with 1/5000 methylene blue in presence of phosphate buffer, p_H 7.4	475
1 cc. <i>B. coli</i> , previously treated with 1/5000 methylene blue in presence of veronal buffer p_H 7.4	29

It was then shown that the toxic effect of methylene blue on *B. coli*-succinate oxidation in presence of veronal buffer could be partly reversed by the subsequent addition of phosphate buffer at the same p_H . This is indicated by the following figures:

Oxygen uptake in 2 hours in mm.³ by:

(a) 1 cc. <i>B. coli</i> exposed to 1/5000 methylene blue solution in presence of phosphate buffer <i>M</i> /25, p_H 7.4, for 30 minutes, succinate alone then being added	505
(b) 1 cc. <i>B. coli</i> exposed to 1/5000 methylene blue solution in presence of veronal buffer, p_H 7.4, for 30 minutes, succinate alone then being added	25
(c) 1 cc. <i>B. coli</i> exposed to 1/5000 methylene blue solution in presence of phosphate buffer <i>M</i> /25, p_H 7.4, for 30 minutes, a mixture of succinate and veronal buffer, p_H 7.4, then being added	538
(d) 1 cc. <i>B. coli</i> exposed to 1/5000 methylene blue solution in presence of veronal buffer, p_H 7.4, for 30 minutes, a mixture of succinate and phosphate buffer, p_H 7.4, then being added	255

The fact that there is a recovery in oxygen uptake when phosphates are added subsequently to the exposure of the organism to the dyestuff in presence of veronal buffer, indicates that the phosphates must be able to remove methylene blue to some extent from combination with the enzyme. This seems the only feasible explanation, for the fact that replacement of phosphate buffer by veronal buffer does not result in a diminished rate of oxidation of succinate by the untreated organism shows that phosphates are not essential to the oxidation of succinate. Moreover veronal cannot have the effect of making methylene blue more toxic, for the addition of veronal to the organism exposed to the dye in presence of phosphate buffer does not result in a diminished rate of oxidation of succinate. There is the possibility that phosphates and methylene blue compete for the same groups in the enzyme but there is no evidence as yet for this.

Other basic dyestuffs such as toluidine blue and malachite green behave in a similar manner to methylene blue. The acid dyestuffs, however, *e.g.* water blue and Congo red, which in phosphate buffer at p_H 7.4 have little or no toxic action on succinate oxidation by *B. coli* have just as little action in veronal buffer at the same p_H .

It seems likely that the action of the phosphate is to combine with the basic dyestuff ions and prevent their combination with the acid groups of the enzyme, or if such combination has been effected, to accomplish a reversal,

by what is tantamount to an eluting action on the adsorbed or combined dyestuff.

If for veronal buffer, p_H 7.4, there be substituted $M/20$ glycine solution brought to p_H 7.4 by the addition of a little alkali, the toxic action of methylene blue on *B. coli* (towards succinate oxidation) is greatly reduced, the oxygen uptake being nine times that which is obtained after exposing the organism to the dyestuff in veronal buffer and about half that which occurs after exposing the organism to the dyestuff in phosphate buffer. Glycine has a protective action similar to, but much less than, that of phosphate at p_H 7.4. Clearly the magnitude of the protective action of the buffer on the organism will be dependent not only on the concentration of buffer ions but on the relative affinities of the buffer ions and the acid groups of the enzyme for the dyestuff. In this connection the work of Hirschfelder and Wright [1930] and of Stearn and Stearn [1930] on bacteriostasis and the equilibria between dyestuff and proteins is of importance.

Oxidations by muscle and brain.

Meyerhof [1917, 1918] showed that the respiration of micro-organisms (staphylococci) was inhibited 10 to 40 % by methylene blue but that the respiration of the same organisms, after exposure to acetone, increased in the presence of methylene blue. Fleisch [1924] demonstrated that the addition of methylene blue to muscle tissue in presence of HCN brought about an increased rate of oxidation of succinate. Barron [1929] found that the addition of methylene blue to living cells (sea urchin, star fish eggs) and red blood cells produced an increased oxygen consumption. It is clear that the dyestuff is able to play a catalytic role in oxidations on account of its reversible nature and the fact that its leuco-derivative can be oxidised by molecular oxygen without the intervention of a dehydrogenase system. The catalytic action of dyestuffs, generally, on the oxygen consumption of living cells is dependent, as shown by Barron and Hoffman [1930], on their reduction potentials and their ability to penetrate into the cell. The results given in Table I show that the acid or basic nature of the dyestuff and its access to (or adsorption or combination with) the centre at which the hydrogen donator is activated influence considerably its effect on oxidations. In these experiments the organism was exposed to the dyestuff for a certain period before the addition of the substrate under investigation.

It has been a usual practice, in investigations on dyestuffs, to add the substrate (*e.g.* glucose) at the same time as the dyestuff to the suspension of cells. If the oxidation of the substrate, under these circumstances, is rapid, there may be insufficient time for the dyestuff to exert a marked toxic action on the enzymes involved and the observed rate of oxidation will not be complicated by this factor. This, however, is only an ideal condition, for circumstances are usually such that the rate of reduction of the dyestuff is not

extremely rapid. The nature of the buffering substances, too, will have a decided influence on the rates of oxidation in presence of the toxic dyestuffs.

When 0.5 g. fresh muscle is exposed to dyestuff prior to the addition of succinate, the experiment being carried out in precisely the same way as has been described for the bacteria, the toxic action of such basic dyestuffs as malachite green and methyl violet becomes apparent. Illustrative results are shown in Table II.

In a typical experiment 0.5 g. freshly minced rabbit muscle was placed in each vessel of the Barcroft apparatus, and phosphate buffer, p_H 7.4 (to give a final concentration of $M/15$), and dyestuff (to give a final concentration of $1/5000$) added. The apparatus was allowed to shake at 37° for 30 minutes, and then succinate (to give a final concentration of $0.067 M$) was added to the right-hand vessel and saline to the left-hand vessel. The rate of oxygen uptake was measured and compared with the control where the muscle was not exposed to dyestuff. It is important in these experiments to have in the control vessels a volume of distilled water equal to that of the dyestuff solution which is placed in the experimental vessels. If normal saline be used instead of distilled water, confusing results are obtained owing to differences in osmotic tension.

In one experiment, for instance, the oxygen uptake by 0.5 g. muscle, unexposed to dyestuff, in presence of succinate was 386 mm.^3 in 2 hours; the oxygen uptake by the muscle, exposed to $1/5000$ malachite green, in presence of the succinate was 150 mm.^3 in 2 hours. When acid fuchsine and Congo red were substituted for malachite green, the oxygen uptakes in 2 hours were 355 and 375 mm.^3 respectively.

Muscle tissue deteriorates, on standing at 0° , at a far greater rate than a suspension of bacteria, so far as the oxidation of succinate is concerned, and this factor is of importance in estimating the effects of dyestuffs. The results with muscle cannot be compared quantitatively with those found with bacteria, for there is a great difference in the quantity of biological material used. Qualitatively, however, there appears to be a parallel between the action of dyestuffs on bacteria and on muscle.

Methylene blue and toluidine blue must be considered separately, for on certain occasions both these dyestuffs have given rise to an increased rate of oxidation of succinate and on others to a decided inhibition. Much depends on the state of the muscle at the time of experiment, and it seems clear that the variability in results is due to the interplay of two factors, (a) a toxic action on the enzymes involved in the oxidation of succinate, leading to inhibition of the rate of oxidation, (b) a catalytic action, due to reduction of the dyestuff and subsequent oxidation, leading to an increased rate of oxidation. If these two effects happen to balance each other, the net effect of the addition of methylene blue is zero—this has occurred on several occasions. On the other hand, on two occasions accelerations of 42 and 46 % have been noted and on another an inhibition of 31 % was found. Much the same has been found with toluidine blue.

If instead of fresh muscle, well-washed muscle be used, methylene blue has a slightly more vigorous toxic action. If, however, using washed muscle, phosphate buffer be replaced by veronal buffer at the same p_H , methylene blue has a very decided toxic action—an effect precisely similar to that found with the bacteria. Thus in one experiment, using well-washed rabbit muscle, methylene blue brought about a 6 % inhibition in presence of phosphate buffer ($M/15$, p_H 7.4) and a 60 % inhibition in presence of veronal buffer (p_H 7.4).

Brain tissue (sheep's whole brain has been chiefly studied) appears to be almost unaffected by the dyestuffs. In no case (see Table II) has an inhibition

Table II. *Percentage inhibition by dyestuffs of succinate oxidation by muscle and brain*

Dyestuff (1/5000)	Muscle	Brain
Methyl violet	30	0
Malachite green	50	0
Gentian violet	15	0
Methylene blue	0	0
Toluidine blue	0	0
Acid fuchsine	0	0
Water blue	4	0
Congo red	3	0

of the oxidation of succinate, due to the exposure of the tissue to dyes, been observed. Slight accelerations, *e.g.* with methyl violet, malachite green, acid fuchsine, toluidine blue, have been noted but these are irregular. For example:

The O_2 uptake in $1\frac{1}{2}$ hours in presence of phosphate buffer, p_H 7.4, and succinate in mm.³

(a) by 0.5 g. sheep's brain	514
(b) by 0.5 g. sheep's brain exposed to 1/5000 methyl violet	560
(c) by 0.5 g. sheep's brain exposed to 1/5000 toluidine blue	571
(d) by 0.5 g. sheep's brain exposed by 1/5000 malachite green	553

The difference between the effects of, say, methyl violet or malachite green, on the oxidations of brain and muscle tissue is very striking. It seems unlikely that the enzymes involved in succinate oxidation in brain are so differently constituted from those in muscle or bacteria that they are not attacked by the basic dyestuffs—it is more reasonable to suppose that the enzymes are protected either by an inability of the dyestuffs to reach the site of the enzymes in the cell or because there are constituents present in the tissue which combine preferentially with them.

Clearly, the locality of the enzymes in the tissues and the nature of their immediate environment are most important factors in determining the toxicity of dyestuffs.

SUMMARY.

1. The action of 29 dyestuffs on the oxidations of glucose, lactate, succinate and formate by *B. coli* has been investigated.

2. It is shown (a) that all the dyestuffs which have a vigorous toxic action are basic in character, whilst the acid dyestuffs have little or no action; (b) that the molecular structure of the dyestuff plays an important part in

determining toxicity; (c) that specificity of behaviour towards the dehydrogenases is exhibited.

3. The toxicity of the basic dyestuffs is greatly dependent on the nature of the buffering agent present.

4. The action of dyestuffs on the oxidation of succinate by muscle tissue is similar to that found with bacteria. In the case of methylene blue or toluidine blue two effects take place, (a) a toxic action leading to inhibition, (b) a catalytic action leading to an increased rate of oxidation, due to the reversible nature of the dye. As in the case of bacteria, the presence or absence of phosphate influences considerably the toxicity of the dyestuff.

5. The dyestuffs, so far examined, appear to have no inhibitory action on the oxidation of succinate by brain tissue.

We are indebted to the Medical Research Council for a grant to this laboratory in aid of equipment and for a whole time grant to one of us (A. H. M. W.).

REFERENCES.

- Bamann and Schmeller (1931). *Z. physiol. Chem.* **194**, 1.
Barron (1929). *J. Biol. Chem.* **81**, 445.
— and Hoffman (1930). *J. Gen. Physiol.* **13**, 483.
Cook and Stephenson (1928). *Biochem. J.* **22**, 1368.
Fleisch (1924). *Biochem. J.* **18**, 294.
Hirschfelder and Wright (1930). *J. Pharm. Exp. Ther.* **38**, 411, 433.
Marston (1923). *Biochem. J.* **17**, 851.
Meyerhof (1917). *Pflügers Arch.* **169**, 87.
— (1918). *Pflügers Arch.* **170**, 367.
Michaelis (1930). *J. Biol. Chem.* **87**, 33.
Quastel and Wooldridge (1927). *Biochem. J.* **21**, 148.
Stearn and Stearn (1930). *J. Exp. Med.* **51**, 341.

LXXIII. THE EFFECT OF ADDING VITAMIN A TO A RACHITOGENIC DIET.

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It is generally held that a direct relation exists between growth-rate and intensity of rachitic lesions in the human subject. Thus Hess [1929] writes, "In general, it may be stated that the more rapid the growth, the greater the tendency to the development of rickets....Animal experiments confirm this general point of view....All investigators who have used the rat as the experimental animal have reported similar experiences." There are abundant references in the literature to "spontaneous healing," and this has in very many cases been associated with a diminished food intake and a retarded rate of growth.

Recently Coward and Cambden [1929] made a special investigation of this matter. They made use of a modified McCollum line test, and did not find any significant correlation between intensity of rickets and failure to grow. Their results were in contradiction to those reported by Bills, Honeywell and MacNair [1928], who remarked "It is imperative to discard all rats which lose even as little as 1 g." Bills also used the line test technique; whereas the curative period in Coward's experiments was 10 days, that in Bills's investigations was only 5. The experiments of Coward, of Bills and of other investigators who have reported results in either sense, were not primarily designed to investigate the particular point under discussion; their conclusions have been drawn from a considerable body of experimental evidence bearing mainly on different points. Bills has recently re-affirmed the view expressed in the earlier paper [Bills, Honeywell, Wirrick and Nussmeier, 1931], and has repeated the exact words of the sentence already quoted.

The following investigation was planned and concluded before the publication of the paper last mentioned. It was designed with the specific object of investigating directly the relationship between increase in growth and intensity of rickets.

The matter is of considerable importance in the assay of vitamin D. The rachitogenic diet most used in this country is Steenbock's 2965. In this the only source of vitamin A is yellow maize. It is known that maize varies in its vitamin A content, the white-cap variety being practically devoid of it [*e.g.* Steenbock and Coward, 1927; Hauge and Trost, 1928]. There is even no reason for assuming that all samples of yellow-cap maize are equally potent

sources of vitamin A. It might therefore happen that a particular batch of diet 2965 was actually deficient in the growth-promoting properties associated with vitamin A (or carotene). If this batch were being used in the comparison of a sample of cod-liver oil with irradiated ergosterol, the presence of vitamin A in the former might bring about markedly greater growth in the animals receiving it than in those receiving the irradiated ergosterol. If this increased growth were associated with greater intensity of rickets, the comparison of antirachitic potency would be thereby completely invalidated. Accordingly the investigation here described was undertaken to determine whether the daily addition of fresh carrot to diet 2965 affected the degree of rickets produced by the diet (*a*) when no source of vitamin D was added and (*b*) in the presence of two different levels of vitamin D supplement.

Thirty-one animals were divided into three groups, the first receiving no source of vitamin D, the second a very small daily dose, in one drop of olive oil, and the third eight times as much as the second, dissolved in the same weight of the same olive oil. The doses administered in the two cases were approximately 0.1 and 0.8 Medical Research Council units daily; no great stress is laid upon the absolute amount of vitamin D administered, but it is definitely known that the two dosages were in the ratio of 1 : 8, since they were made up from the same preparation of irradiated ergosterol. Each group was divided into two sub-groups, in one of which each animal received 1 g. of fresh carrot daily. Although no assay of vitamin A (or carotene) was carried out on the samples of carrot used, similar samples purchased at the same time were found to be ample in daily doses of 1 g. to restore growth to animals "run out" on a vitamin A-free diet. In view of the unanimity of the literature on the subject, it can safely be assumed that the animals receiving the supplementary doses of carrot were receiving adequate supplies of vitamin A.

The experimental period was 5 weeks; the animals were all kept in separate cages. One hind-leg and one fore-leg of each animal were taken for extraction and ashing, and photographic records were kept of the "lines" from the other tibia of each animal. In general the appearance of the lines was quite consistent with the figures obtained by chemical analyses. The criterion of rickets used in these experiments has been the percentage of ash in the moisture- and fat-free bones, [A]. This figure is closely related to the "rachitic index," used by Chick and Roscoe [1926], which is the ratio of the ash to the non-fatty organic matter in the bone. This last figure, denoted R, is related to [A] and to R.I. (the rachitic index) in the following way:

$$R.I. = \frac{[A]}{R} = \frac{[A]}{100 - [A]}$$

When the value of [A] is 50, the R.I. is clearly 1.

Records were kept of the initial and final weights of all the experimental animals. As far as possible animals of the same sex and from the same litter (for which it is proposed to use the term isogenic animals) were distributed over the various dosages administered.

The actual increases in weight appeared to bear no relation to the weights of the animals at the beginning of the experimental period, but rather, as was to be expected, to show some correlation with the individual litters. Since the intensely inbred London strain of Wistar rat was used throughout, the differences can hardly have been genetic, and are probably associated with differences of prenatal nutrition and fat-soluble vitamin storage.

The value for [A] was obtained on the bones after they had been extracted with alcohol to constant weight. We have found this to be an essential precaution, since the size and porosity of different bones are the main factors affecting the ease with which the fat and moisture can be removed from them. We have also found that subsequent ether extraction is unnecessary since it never causes a further loss in weight of more than 1 or 2 mg. The bones were ashed in a muffle at dull red heat, and were also weighed a second time after a second heating, although the latter never produced any significant change in weight.

Table I shows the distribution of litters, animals, and doses, the value found for [A] and for the increase in growth during the experimental period, δM (in brackets).

Table I. *Values for [A] and δM (in brackets) for 31 animals from 3 litters.*

		Vitamin D					
		No vitamin D		0.10 unit		0.80 unit	
Litter		No carrot	Carrot	No carrot	Carrot	No carrot	Carrot
1534	7 ♂	40.9 (48)	45.5 (62)	42.3 (60) 37.0 (47)	39.8 (52)	44.4 (59)	46.5 (45)
	7 ♀	34.2 (41)	34.2 (33)	34.8 (42)	38.7 (44) 46.4 (32)	40.1 (47)	45.1 (43)
1529	7 ♀	33.5 (22)	32.8 (31) 32.3 (38)	40.1 (38)	42.3 (40)	41.5 (38)	44.2 (35)
1386	6 ♂	38.2 (74)	36.4 (72)	38.8 (78)	28.4 (63)	41.6 (80)	48.4 (86)
	4 ♀	33.4 (44)	31.7 (51)	39.1 (58)	—	36.1 (45)	—
Averages		36.0 (46)	35.5 (48)	38.7 (54)	38.7 (46)	40.7 (54)	46.1 (52)
		35.8 (47)		38.7 (50)		43.4 (53)	

DISCUSSION OF RESULTS.

It appears that the batch of diet 2965 used in these tests was an adequate source of vitamin A, under the conditions of the experiment, since a daily supplement of 1 g. of carrot did not appear to bring about a significant change in growth-rate. The average increases for all animals during the whole 5 weeks were:

without carrot (7♂, 9♀) 51
with carrot (6♂, 9♀) 48

The difference between the averages is not more than could be accounted for by the slightly different sex-ratio and other normal variations and is certainly not significant.

It also appears that there is no correlation whatever, under the conditions and within the limits of this experiment, between growth-rate and severity of rickets, as chemically assayed and corroborated by the line test technique. Fig. 1 shows [A] plotted against δM for all the animals.

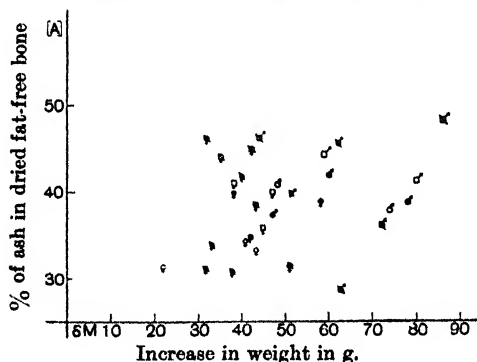


Fig. 1.

Without carrot.		With carrot.	
○	without vitamin D.	◊	without vitamin D.
●	with 0.1 unit vitamin D.	◐	with 0.1 unit vitamin D.
◻	with 0.8 " "	◑	with 0.8 " "

The carrot appears to have exercised no antirachitic effect when given unsupplemented at a daily level of 1 g. There is a suggestion that higher, and nearly prophylactic, doses of vitamin D are made more effective in the presence of increased vitamin A (or carotene), but the number of animals used was insufficient, and the differences in bone-ash percentage too fluctuating, for this to be anything more than an indication. Nevertheless, all four relevant pairs of isogenic animals show this effect, and its significance seems probable. Steenbock *et al.* [1930], amongst others, have called attention to the absence of evidence that "the various factors operative in bone production act independently and consistently without one modifying the influence of the other."

SUMMARY.

1. Addition of 1 g. of fresh carrot daily to the diet of animals on Steenbock's diet 2965 did not significantly affect growth-rate, whether in the absence or presence of vitamin D.

2. No evidence was found, under the conditions of the experiment, for any correlation between severity of rickets and rate of growth.

REFERENCES.

- Chick and Roscoe (1926). *Biochem. J.* **20**, 1.
 Coward and Cambden (1929). *Quart. J. Pharm.* **2**, 44.
 Bills, Honeywell and MacNair (1928). *J. Biol. Chem.* **76**, 251.
 ——— Wirrick and Nussmeier (1931). *J. Biol. Chem.* **96**, 619.
 Hess (1929). Rickets, osteomalacia and tetany, 247 (Philadelphia).
 Hauge and Trost (1928). *J. Biol. Chem.* **80**, 107.
 Steenbock, Black and Thomas (1930). *J. Biol. Chem.* **85**, 596.
 ——— and Coward (1927). *J. Biol. Chem.* **72**, 765.

LXXIV. THE INFLUENCE OF CALCIUM ON THE RATE OF DIFFUSION OF SUGARS THROUGH SURVIVING INTESTINE.

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(Received March 9th, 1931.)

In previous work on this subject [Macleod *et al.* 1930; Auchinachie *et al.* 1930], the fluid bathing the surviving segments of intestine was always sugar-free Tyrode's solution. In view of the well-known effects of electrolytes on the physiological activity of living cells, it was decided to determine whether the selective properties of the intestinal epithelial cells would be affected by varying the composition of the outer fluid.

The procedure was the same as in previous experiments except that, as it was necessary to prepare the segments in sugar-free but otherwise normal Tyrode (called "normal Tyrode" in text), each segment was thoroughly washed in Tyrode of the composition to be used for the test, so as to ensure that all traces of fluid, except that whose influence was to be studied, would be removed from the intestine. Rabbit's small intestine was used, and its generally greater permeability in an aboral direction was ruled out by suitable selection of segments for control experiments. The volume of outer fluid was 50 cc., and 4 cc. 0.75 *M* sugar solution were generally placed in the lumen.

RESULTS.

When the rates of diffusion of 0.75 *M* glucose through the walls of segments bathed in normal and in Ca-free Tyrode respectively were compared, the rate was found to be greater in the former case, as the following figures from a typical experiment show:

Sol.	45	90	135 mins.
Ca-free Tyrode	29	103	185} mg. glucose per
Normal Tyrode	49	137	275} 100 cc. fluid

The segments bathed in Ca-free Tyrode showed scarcely any movements; whereas those bathed in normal Tyrode contracted vigorously and undoubtedly increased the pressure of the lumen contents. This factor was not, however, responsible for the more rapid diffusion, because inhibition of the movements by continuous slow addition of a weak adrenaline solution did not alter the nature of the result. By starting with all the segments immersed in Ca-free Tyrode and then adding the usual amount of CaCl_2 to one series of beakers,

after a suitable interval, the accelerating effect of the CaCl_2 on diffusion was always elicited (see Fig. 1). When the diffusion was allowed to proceed into

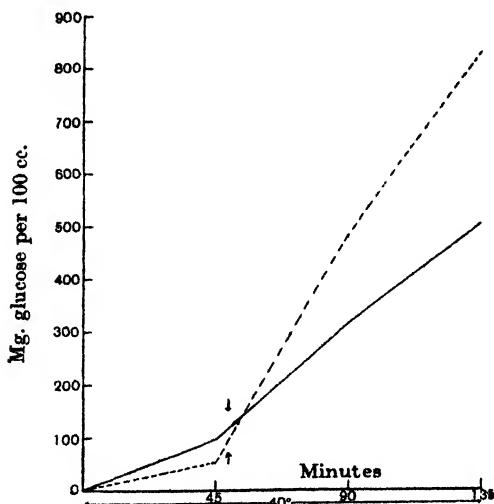


Fig. 1.

— Ca in outer fluid. Oxalate added at ↓.
 - - - No Ca in outer fluid. Ca added at ↑.

normal Tyrode for a period and the Ca then precipitated by the addition of an equivalent amount of oxalate to one beaker a slowing of the rate of diffusion occurred (see Fig. 1). Similar results were obtained by adding an equivalent amount of citrate instead of oxalate. This result shows that the accelerating effect of calcium on the diffusion of glucose is due to calcium ions and not to un-ionised calcium, since the citrate, while it removes Ca^{++} , leaves un-ionised calcium in solution.

We are not aware of any reason why Ca^{++} should increase the permeability of the intestinal wall. Indeed, a contrary effect would not have been surprising in view of the discussion of the literature in an earlier paper [Magee and Reid, 1927]. The foregoing results having suggested that Ca^{++} was necessary for the physiological phenomena manifested by surviving intestine, it became all the more necessary to perform experiments testing this conception more stringently.

Effect of temperature. Adjacent segments containing the same volume of 0.75 M glucose were suspended in Ca-free and in normal Tyrode respectively and kept at 0° for a period. After withdrawing samples from the outer fluid the temperature was suddenly raised to 40° . Fig. 2 shows a typical result. It will be seen that after 30 minutes at 0° the amounts of glucose that diffused out were approximately equal, whereas at 40° the rate of diffusion was much more rapid in the case of the segment bathed in normal Tyrode. Since all physiological activity was in abeyance at 0° the equality in the rates of diffusion may be taken as proof that Ca^{++} would not affect the diffusion of glucose, either on

account of attraction of sugar by Ca^{++} in the outer fluid, or through an action on the texture of the gut wall. The more rapid diffusion in the case of the segment bathed in normal Tyrode at 40° , therefore, indicates that the physiological activity evoked by the rise in temperature was only possible in the

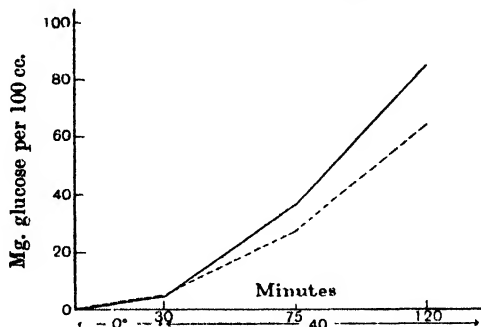


Fig. 2.

———— Ca in outer fluid. - - - - - No Ca in outer fluid.

presence of Ca^{++} . The results given below show that it made little difference to the rate of diffusion whether the Ca^{++} were present in the inner or outer fluid.

Inner fluid	Outer fluid	30	60	90 mins.	
4 cc. 0.75 M glucose	Ca-free Tyrode	17.1	43.4	80.5	} mg. glucose per 100 cc. outer fluid
4 cc. 0.75 M glucose in 0.02 % CaCl_2	Ca-free Tyrode	21.0	50.3	101.2	
4 cc. 0.75 M glucose	Normal Tyrode	16.6	50.9	107.0	

Comparison of glucose and xylose. Previous work showed that, when equimolecular solutions of glucose and xylose were placed in adjacent loops, glucose generally passed into the outer (normal) Tyrode more rapidly than xylose. On repeating these experiments with Ca-free Tyrode no evidence of selective uptake of glucose could be demonstrated, but instead xylose passed out more rapidly than glucose, as was found in the case of dead intestine. This result again shows that the surviving intestine requires Ca^{++} to enable it to discriminate between the physiological sugar, glucose, and the foreign sugar, xylose.

Different concentrations of sugar. When equal volumes of M and 0.75 M glucose were placed in adjacent loops of surviving intestine, suspended in Ca-free Tyrode, diffusion was more rapid in the case of the M solution. This result, which is in accordance with the diffusion laws, is contrary to those previously obtained for diffusion into normal Tyrode. In this case diffusion was maximal with 0.75 M solutions.

It will, therefore, be seen that when the surviving intestine is examined by the above-described methods it fails to display in the absence of Ca^{++} those selective properties which are shown in the presence of Ca^{++} . Further experiments suggest, however, that Ca^{++} is not the only ion whose presence is

necessary for the manifestation of this selective action. Thus, when the diffusion of glucose was compared with segments bathed in normal and K-free Tyrode, the rate was greater in the former case.

Sol.	30	60	90 mins.
Normal Tyrode	39	91	154) mg. glucose per
K-free Tyrode	38	87	139) 100 cc. outer fluid

Similarly, diffusion was more rapid when the outer fluid was normal Tyrode of p_H 7.4–7.6 than when this was of p_H 5.4. The promotion of diffusion by the higher p_H was also shown to be independent of the concentration of HCO_3^- . But further work will be necessary before the rôles played by these ions in absorption can be clearly stated.

This investigation as a whole also shows that the passage of glucose through the wall of surviving intestine into an outer environment of Tyrode's solution is, to some extent, of the nature of an active transport of sugar molecules by the activity of the epithelium as well as a process of simple diffusion. But, as stated in an earlier paper, the effect of the surviving activity of the cells is liable under severe test to be masked by the purely physical phenomena.

SUMMARY.

In experiments with surviving rabbit's intestine it has been found that glucose diffuses more slowly from the lumen into Ca-free than into Ca-containing outer fluids, the process in the former case being a physical one but in the latter partly of the nature of a selective action.

The presence of calcium ions in the environment is indispensable for the manifestation of selective action of the intestinal wall towards sugars placed in the lumen.

We desire to express our thanks to Professor Macleod for advice and criticism.

REFERENCES.

- Auchinachie, Macleod and Magee (1930). *J. Physiol.* **70**, 185.
 Macleod, Magee and Purves (1930). *J. Physiol.* **70**, 404.
 Magee and Reid (1927). *J. Physiol.* **63**, 97.

LXXV. ON THE INFLUENCE OF SERUM ON ENZYMES, WITH SPECIAL REFERENCE TO ITS ACTION ON TRYPSIN.

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Introduction.

THE influence of serum on enzymes may be considered kinetically as falling into one of two categories. Either the enzyme is influenced by the presence of a similar enzyme in serum, in which case an apparent acceleration is produced, or else the action is associated with an absence from serum of the enzyme in question, in which case the action is one of true acceleration, or inhibition, as the case may be; the mere fact that serum accelerates or inhibits an enzyme constitutes in itself incomplete evidence of the nature of the action. In a recent publication [1930], I have shown how failure to recognise this principle has already vitiated work done on the subject.

It is usually a simple matter to determine whether an apparent acceleration is due to the presence of the enzyme in serum, or whether the acceleration is a true one; greater discrimination however is necessary to establish the presence of both factors if present simultaneously in a case of acceleration, and to determine to what extent each is responsible for the effect observed.

Serum-enzymes. In some instances the action of serum is wholly explained by the presence of a serum-enzyme, as in the case of serum-diatase; in other cases the action is only partially explained by the presence of an enzyme in serum, an example of which is human serum acting on lipase.

The enzymes recorded in serum include protease, peptidase, diastase, lipase, catalase, invertase, and maltase. It is important to recognise that the distribution of enzymes in serum is by no means identical in different species.

Thus, while diastase is fairly widespread, occurring in man, sheep, cow, rat, and guinea-pig, it is absent from rabbit [Luers and Albrecht, 1926]. Lipase I have also found in man, sheep, cow, rat and guinea-pig. Maltase was not found in guinea-pig serum [Blacklock, Gordon and Fine, 1930] but has been recorded in pig serum. Invertase I have failed to find in a number of animals, including man, but under experimental conditions (*e.g.* following intraperitoneal injections of a commercial preparation of invertase) I have

found it in guinea-pig serum, where it persisted for over 30 hours in one instance [Fine, 1930].

The influence of serum on enzymes not ascribable to the presence of serum enzyme. It is this category of the influence of serum on enzymes that is the subject of the present paper.

Even when serum does contain an enzyme, its action may be greater than is to be accounted for by the presence of the enzyme; for example, human serum will increase the activity of lipase to a degree exceeding what would be expected from the mere addition of a lipase of the strength of serum-lipase. The total acceleration is, in this case, partly due to the presence of serum-lipase (apparent acceleration), and is partly a true acceleration.

It has been customary to explain the effect of acceleration by serum as due to "an accelerator," and the effect of inhibition as due to an "inhibitor," or, more commonly, an "anti-enzyme." I have shown however [1930] that in the case of invertase the influence exerted by serum is entirely a function of p_H , the serum acting as an accelerator over certain ranges of p_H and as an inhibitor over others.

In the following pages I have brought together evidence both from my own experiments and from the work of others, which justifies the conclusion that with most other enzymes also true acceleration or inhibition by serum is not due to specific accelerators or anti-enzymes, but to various physical conditions such as p_H or the adsorption of the enzyme by serum, the latter being particularly significant in the case of the antitryptic action of serum.

In the previous publication referred to [1930] I have already dealt with the enzyme-accelerating properties of serum, and there pointed out that serum has been known to accelerate only two enzymes—invertase and lipase. The same serum which accelerates invertase will also inhibit it if the p_H is suitably altered. The influence of p_H on the lipase-accelerating power of serum has not been worked out.

Anti-enzymes reported in serum.

The origin and significance of the term "anti-enzyme." The first observation of the phenomenon of enzyme-inhibition by serum was made by Hildebrandt [1893], who discovered that serum inhibited the action of emulsin. Hahn [1897] followed with the observation that serum inhibited trypsin, and Morgenroth [1899] found that it inhibited rennin.

Although none of these workers isolated any substances from serum which might account for the inhibition observed, they all explained the phenomenon observed by the presence of corresponding anti-enzymes in normal serum. Very early, however, Landsteiner [1900] showed, and Cathcart [1904] and others confirmed, that trypsin inhibition was a property of the albumin fraction of serum rather than of the globulin fraction, whereas it was well established that the immunological antibodies resided in the globulin fraction.

Furthermore no one could demonstrate a material increase of the enzyme-inhibiting action of serum following enzyme injections. The consensus of opinion appears to be that trypsin is the only enzyme that can evoke the response of an increased enzyme-inhibition in serum, and even so, the increase never exceeds three or four times the original amount of inhibition. Such response is to be sharply differentiated from the effects of toxin injection on antibody formation in serum: true antibodies can invariably develop to the amount of thousands of times the original antibody content.

Weil [1910] pointed out that while serum can inhibit the haemolytic action of saponin, it does not necessarily follow that serum contains an antisaponin, and, indeed, as Bayliss [1919] says, if a substance which inhibits an enzyme is entitled to be called an anti-enzyme, then sodium hydroxide is antipepsin.

The action of serum on enzymes is largely a function of p_H . The earlier workers, as Wells [1925] has pointed out, seem to have quite ignored the effect of p_H . Bayliss [1912] in examining the evidence for Hildebrandt's anti-emulsin found that the mere addition of serum to an emulsin mixture sufficiently altered the p_H to produce a corresponding fall in activity of the emulsin. Bensley and Harvey [1912] showed that the absence of free HCl from the cells of the gastric mucosa formed the best protection against the secreted pepsin: in other words, the p_H of the cell substance was in itself an adequate "antipepsin." Dragsted and Vaughan [1924] sewed the spleen and kidney into openings made in the stomach wall, and found that they were unaffected by the consequent exposure to pepsin, provided the blood supply were kept intact. In their view the mechanism of this immunity was simply the buffer action of the blood and the absorption of the HCl and pepsin into the bloodstream. Thaysen [1915] found that "antirennet" could also be accounted for by the change in p_H when serum is added to a rennet mixture: he also found that adsorption of rennet by the serum-proteins played an additional part. The previously mentioned observations of the present author [1930] on invertase afford strong evidence in the same direction.

In the case of other enzymes however the influence of serum under different conditions of p_H has not hitherto been worked out, and although p_H regulation must play some part, it is probable that a number of factors are involved in the defence of the organism against the action of excessive amounts of enzyme. Excretion is one of the chief factors, as in the case of amylase; destruction in the body probably occurs in the case of invertase [Fine, 1930]; adsorption by tissue- or serum-proteins in the case of trypsin.

At various times serum has been reported to possess an inhibitory action towards trypsin, pepsin, rennin, papain, "autolytic enzymes," lipase, amylase, invertase, laccase, catalase, tyrosinase, emulsin, urease and fibrin-ferment. Consideration of the p_H optima of these enzymes shows however that the mere addition of serum which has a p_H of 7.45 would greatly reduce the activity of unbuffered solutions of pepsin, rennin, papain, autolytic enzymes, emulsin, and invertase. Even invertase, which is accelerated by serum when buffered

at its optimum p_H , is totally inhibited when serum is added to its unbuffered solution.

In the case of trypsin however, the p_H factor does not appear to play any part, the inhibition of trypsin by serum being, as will be shown later, purely a manifestation of the power of serum to adsorb the enzyme. Occupying an intermediate position between pepsin on the one hand, and trypsin on the other, there is a group of enzymes, of which rennin is an example, whose inhibition by serum is partly a p_H effect and partly an adsorption effect [Thaysen, 1915].

Recovery of trypsin from inhibition by serum.

Following Hahn's observation [1897] that serum contained antitrypsin, *i.e.* retarded the action of trypsin, many substances of organic nature were examined for their influence on trypsin, in the hope of some light being thrown on the phenomenon. Among these so-called inhibitors may be mentioned (1) extract of intestinal worms [Weinland, 1903; Dastre and Stassano, 1903; Hamill, 1906; Burge, 1915]; (2) egg-white [Vernon, 1904; Sugimoto, 1913; Bayliss, 1923]; (3) bacteria [Czapek, 1903; Jobling and Petersen, 1914, 4]; (4) yeast [Buchner and Haehn, 1910]; (5) body tissues generally [Levene and Stookey, 1903]; (6) cells of gastric mucosa [Blum and Fuld, 1906; Langenskiold, 1914]; (7) mucous secretion [Langenskiold, 1907]; (8) erythrocytes [Czapek, 1903]; (9) inflammatory exudates [Opie, 1905]; (10) urine [von Schoenbom, 1910; Fujimoto, 1918]; (11) unsaturated fatty acids [Jobling and Petersen, 1914, 1]; (12) lecithin [Schwartz, 1909].

An examination of the properties of these various substances showed, however, that the mechanism of their inhibition of trypsin had little in common, and that egg-white only may be considered as acting in a similar manner to serum. Among the various properties in which these two substances differed outstandingly from other trypsin-inhibiting bodies was the temporary nature of the inhibition. This property was however shared by worm extract whose behaviour generally otherwise differentiated it clearly from serum-antitrypsin.

Experimental demonstration of recovery of trypsin.

All the methods of estimating antitrypsin hitherto employed—Gross [1908] (casein precipitation); Folin and Denis [1911] (incoagulable nitrogen); viscometric method [Feldstein and Weil, 1909–10]; electro-conductivity method [Bayliss, 1923]; refractometric method [Robertson, 1918]—are subject to the criticism that when two digesting mixtures are compared, one containing serum and the other containing an equal amount of water, but otherwise the same in composition, the amount of protein in the two tubes is not the same, and therefore the amount of digestion not strictly comparable; for it must be remembered that serum is a 6 % protein solution. The technique I have used and found highly satisfactory has been as follows.

Two tubes were prepared containing equal quantities of trypsin, buffer (p_H 8), and serum; in the first the serum had been heated at 70° for half an hour to destroy antitryptic action and in the second the serum was untreated. An aliquot portion of each tube was titrated by Sørensen's method immediately the constituents were added and mixed and a fixed number of hours later. The rise in acidity indicated the amount of tryptic action that had taken place in the two tubes containing the same concentration of trypsin and substrate. If the rise in acidity in the first tube is A , and that in the second B , then the ratio $\frac{A-B}{A}$ adequately measures the antitryptic activity.

One of the chief advantages of the Sørensen method is the additional information it gives when the free and "formol" acidities are considered separately.

Using the Sørensen method and the technique I have outlined I was able to follow the course of digestion of serum-protein by trypsin, and in this way not only obtained the Dastre [1903] effect with serum, *i.e.* enzyme recovery following preliminary inhibition, but found that almost invariably the recovery of the trypsin from inhibition was such that the inhibited trypsin ultimately showed greater activity than the trypsin acting freely on heated serum.

The experiments showing this recovery were carried out as follows:

Two tubes were incubated whose contents were as below:

	Tube 1	Tube 2
	cc.	cc.
Trypsin	0.2	0.2
Serum	0.1	0.1 (inactivated)
Buffer p_H 8	0.7	0.7

(a) The source of trypsin was Liquor trypsin co. (Benger's) and before use it was neutralised with $N/10$ NaOH to p_H 8 and then further diluted to 50 % of original strength.

(b) The buffer was prepared according to Clark and Lubs.

(c) The serum in tube 2 was inactivated by heating at 70° for half an hour. It was heated together with the buffer so as to avoid the difficulty of mixing afterwards; during the heating the tube was stoppered to avoid loss of volume by evaporation.

(d) After tube 2 was cooled, the trypsin was added to the two tubes from which, after adequate mixing, 0.1 cc. was immediately withdrawn for titration of acidity with $N/100$ NaOH. A layer of toluene was added to the tubes before incubating.

The method of titration was that employed by Wigglesworth [1928] in his investigation of cockroach protease. The pipetted fluid (0.1 cc.) was added to 5 cc. of distilled water in a test-tube; 5 drops phenolphthalein solution (0.05 % in 50 % alcohol) were added, and $N/100$ NaOH was added from a 2 cc. microburette graduated in 0.01 cc. The alkali was added until a pink colour developed identical with that of a tube (of similar make) containing

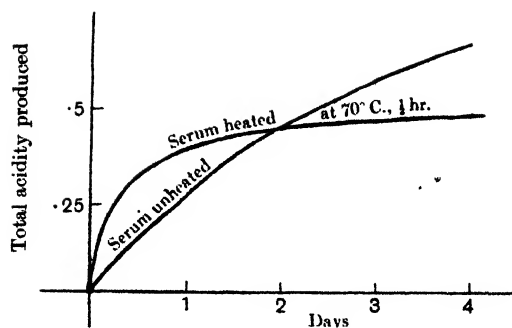


Fig. 1. Digestion of 10 % guinea-pig serum by 10 % trypsin. This shows recovery of inhibited trypsin until activity is greater than that of uninhibited trypsin.

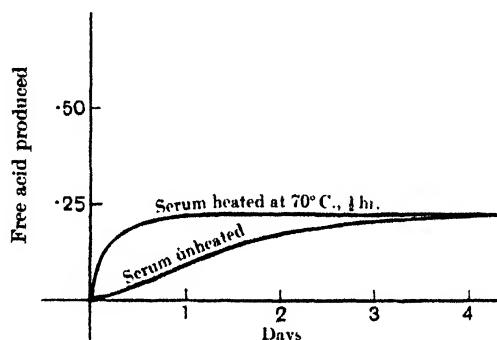


Fig. 2. Free acid produced in digestion of 10 % serum by 10 % trypsin. This shows how free acid produced by inhibited trypsin ultimately equals amount formed by uninhibited trypsin.

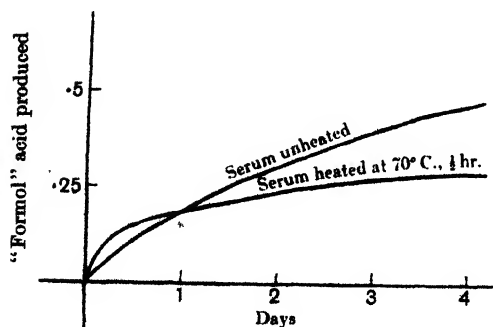


Fig. 3.

Fig. 3. "Formol" acid produced in digestion of 10 % serum by 10 % trypsin. This shows how inhibited trypsin ultimately forms more "formol" acid than uninhibited trypsin.

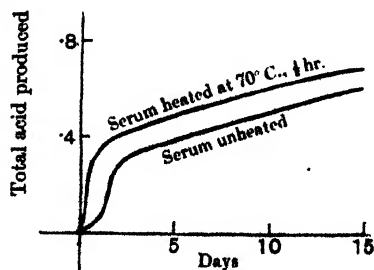


Fig. 4.

Fig. 4. Total acid curves of digestion of 10 % serum by 10 % trypsin. Recovery of inhibited trypsin is incomplete in this case: the serum was obtained from a guinea-pig which had recently had a large injection of trypsin.

5 cc. of buffer solution of p_H 9 plus 5 drops of 0.05 % phenolphthalein in 50 % alcohol. The end point was remarkably sharp.

The figure thus obtained was a measure of the "free acid" present; the "formol acid" was obtained by adding 0.5 cc. of 50 % neutralised formalin (p_H 8) and titrating further with alkali till the end point was reached again. When desired the free and "formol" acids were combined in a single figure—the "total acid."

Determinations of the acid present in 0.1 cc. of the incubated mixtures were carried out from time to time, and in this way curves of digestion were plotted.

Fig. 1 is the curve of a typical experiment, showing how the initial inhibition of trypsin is finally replaced by digestion more active than in the tube where trypsin was not initially inhibited. An analysis of Fig. 1 in the form of separate curves of free and of "formol" acid formation (Figs. 2 and 3) throws some light on the phenomenon. The latter figures show that the final superior digestion of unheated serum applies only to "formol" acid formation: on no occasion have I found more free acid ultimately formed in the presence of unheated serum (tube 1) than of heated serum (tube 2), whereas "formol" acid formation was almost invariably greater in tube 1 than in tube 2, provided digestion was sufficiently prolonged.

In some cases, even after weeks of incubation, the total acid in tube 1 remained below the value in tube 2. An example of this type of incomplete recovery is shown in Fig. 4 the curve of which was obtained from the serum of a guinea-pig which 7 days previously had received a large injection of Liquor trypsin co. An analysis of the total acid curve, in Figs. 5 and 6, shows that the incompleteness of recovery of the trypsin affects only the free acid formation, the "formol" acid curves intersecting in the same way as in Fig. 3.

Figs. 7, 8 and 9 are the total, free, and "formol" acid curves of digesting mixtures containing

10 % gelatin	0.5 cc.
Trypsin 50 %, neutral	0.5 cc.
Buffer p_H 8.5...	0.5 cc.
Serum...	0.2 cc.
Saline 0.88 %	0.3 cc.

Here also the greater digestion of unheated serum affects the "formol" acid curve only. The addition of the gelatin appears to hasten the recovery of the trypsin.

Interpretation of foregoing results. (a) It would appear on examining Fig. 1 that serum can not only retard the action of trypsin, but can at a later stage enhance it, after passing through an intermediate stage during which trypsin becomes gradually released from its bonds. In the Fig. 4 type of experiment the stage of enhancement is not reached, though the trypsin has recovered considerably from the inhibiting action of serum.

(b) An examination of the free and "formol" acid curves separately shows very strikingly that the transition from inhibition to enhancement applies

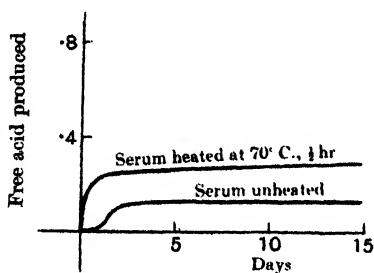


Fig. 5.

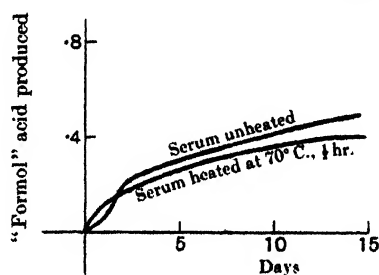


Fig. 6.

Fig. 5. Free acid curves of digestion of 10 % serum by 10 % trypsin. This experiment is the same as in Fig. 4, the free acid only being considered.

Fig. 6. "Formol" acid curves of digestion of 10 % serum by 10 % trypsin. This experiment is the same as in Fig. 4, "formol" acid only being considered.

only to the "formol" acid formation; the extent of formation of free acid with unheated serum never exceeds that with heated serum although, provided

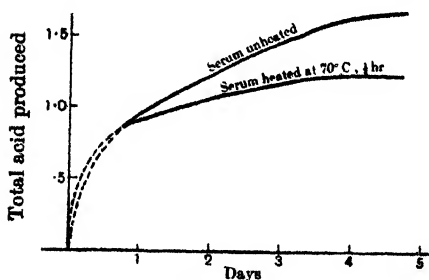


Fig. 7.

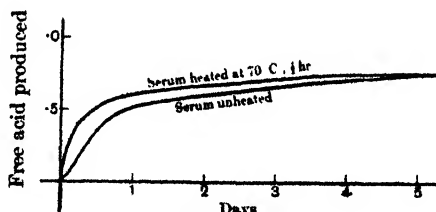


Fig. 8

Fig. 7. This shows accelerated recovery of inhibited trypsin in presence of gelatin. The dotted part of the curves is hypothetical, as no estimation of acidity was made during that interval.

Fig. 8. Free acid curves (2.5 % gelatin present). This shows the same relation between the free acid formed by inhibited and by uninhibited trypsin as in Fig. 2.

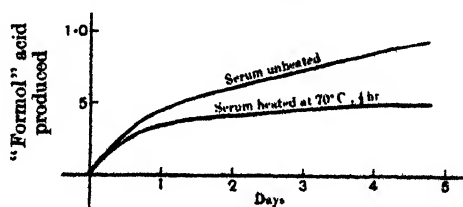


Fig. 9. "Formol" acid curve (2.5 % gelatin present). This shows the same relation between "formol" acid formed by inhibited and uninhibited trypsin as in Fig. 3.

digestion is sufficiently prolonged and the trypsin is fairly active, the same amount of free acid is finally produced in the two tubes: Fig. 5 shows a marked but not complete free acid recovery in the unheated serum tube.

(c) Serum therefore possesses the property, destroyed by heat, of temporarily retarding formation of free and "formol" acid by trypsin, and of subsequently accelerating "formol" acid formation only.

How is this behaviour of serum to be explained? The explanation would prove a difficult matter if it were assumed that pancreatic trypsin, which is the basis of the commercial preparation used (Liquor trypsin co.) were a pure enzyme. The pancreatic secretion however contains two proteolytic enzymes, protease breaking proteins down to polypeptides and increasing predominantly the free acidity of the digest, and peptidase, hydrolysing the polypeptides to amino-acids and producing a rise in "formol" acidity.

If this view is correct, the behaviour of serum becomes more easy to explain. It does not exert its action against trypsin as a whole, but only against the protease portion of it. The inhibition of protease will, by preventing the liberation from the proteins of a suitable substrate for peptidase, produce an apparent inhibition of the latter. There does not, however, appear to be any necessity for assuming that the initial depression of "formol" acid formation is due to an antipeptidase action of serum rather than to a shortage of substrate consequent on protease inhibition.

But why should "formol" acid production in the unheated serum tubes exceed that in the heated tubes? There are two possible explanations. Serum contains either a peptidase or a peptidase-accelerator. The most likely explanation is the existence of a serum-peptidase, which has been reported by various workers, including Jobling *et al.* [1912] who report that serum-peptidase is destroyed by heating at 70° and [1915, 2] that "serum-peptidase is not influenced by antitrypsin." Hence, when protease has recovered from the inhibition by serum, the peptidase of the unheated serum will reinforce that of the pancreatic trypsin, and so produce "formol" acid at a rate which will outstrip the "formol" acid production in the tube containing heated serum (where pancreatic peptidase acts alone, the serum-peptidase being destroyed by heat).

(d) In a word, the behaviour of serum to pancreatic trypsin can be explained by the presence of two factors, firstly, an antiprotease factor, and secondly, peptidase.

This conclusion is of some importance for at least two reasons. In the first place the evidence justifying it confirms in a novel yet definite manner the dual nature of trypsin; secondly, this evidence, by showing that it is the protease only (the portion forming free acid) which serum inhibits, directs serious criticism against those methods of measuring "antitrypsin" which estimate together the products of both protease and of peptidase actions. The methods thus open to criticism include those of Bayliss (electrometric), and Robertson (refractometric) though not of Folin (uncoagulated N). The error involved will depend on the period of incubation used as well as the strength of serum-peptidase present; the extent of the error will be appreciated in the following example.

Course of digestion of heated and unheated sera									
	8 hrs.			27½ hrs.			95 hrs.		
	F	For	T	F	For	T	F	For	T
Unheated sera	0.03	0.06	0.09	0.13	0.19	0.32	0.23	0.41	0.64
Heated sera	0.22	0.10	0.32	0.27	0.17	0.44	0.27	0.28	0.55
F = Free acid.			For = "Formol" acid.			T = Total acid.			

Figures are cc. N/100 acid in 0.1 cc. of incubated fluid.

It will be readily seen that there is a considerable discrepancy between the degree of inhibition as measured from the free acid and that measured from the total acid. Thus:

	% inhibition (or "antitryptic index")		
	8 hrs.	27½ hrs.	95 hrs.
From free acid figs.	86	52	15
From total acid figs.	72	27	-16*

* i.e. 16 % acceleration.

The methods criticised will therefore give too low a value for the index of inhibition.

The kinetics of serum antitrypsin.

(a) *The kinetics of recovery.* The Dastre phenomenon was only obtained when the concentration of serum was suitably adjusted to the concentration of trypsin. On the other hand, it was found that (1) when the concentration of trypsin was greatly in excess of the serum the latter failed to produce any appreciable inhibition; (2) when the concentration of serum was greatly in excess of the trypsin, the latter completely failed to recover from the inhibition. The correlation of the relative trypsin-serum concentration with the manifestation of the Dastre phenomenon is illustrated by Fig. 10. The curves were

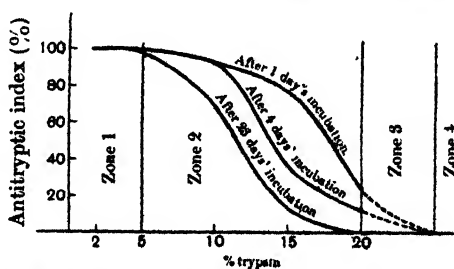


Fig. 10. Inhibition of trypsin (from 2 % to 20 %) by 10 % sheep serum. This illustrates the kinetics of recovery of trypsin from inhibition. The figure suggests a division of the curves into four trypsin zones.

Zone 1. 0 %–5 % trypsin. Here there is no recovery from the inhibiting action of 10 % serum.

Zone 2. 5 %–20 %. Inhibition is initially complete to partial, and recovery is partial to complete.

Zone 3. Trypsin 20 %–25 % (dotted lines); inhibition is initially small, and recovery is complete.

Zone 4. Trypsin over 25 %. Inhibition is absent. No experiments were actually carried out in Zones 3 and 4, which are merely strongly suggested by the character of the Zones 2 and 3, and by the fact established on other occasions that concentrated trypsin successfully resists the inhibiting action of serum.

obtained by plotting the digestion of a series of tubes containing (1) trypsin in concentrations from 20 % to 2 % of the commercial preparation; (2) sheep serum in constant concentration of 10 %, and (3) buffer, p_H 8. A pair of tubes was put up at each concentration of trypsin, one containing unheated serum (tube 1), the other heated (tube 2): each point on the curve was obtained by expressing as a percentage the ratio

$$\frac{\text{total acid in tube 2} - \text{total acid in tube 1}}{\text{total acid in tube 2}}$$

which expresses the degree of inhibition by the unheated serum. The total acid figures were taken because in this case the value of serum-peptidase was practically nil, so that the error involved in including "formol" acid did not affect the nature of the curves obtained, although it would affect the accuracy of the individual antitryptic indexes.

An examination of Fig. 12 shows that (1) in concentrations of 4 % and less trypsin is completely inhibited and does not recover from the inhibition even after 23 days; (2) between 4 % and 20 % trypsin is only partially inhibited and recovers to an extent proportional to its concentration; (3) at 20 % recovery is ultimately complete. From the nature of the curves it would be reasonable to infer that concentrated trypsin completely resists the inhibiting action of serum (dotted lines in Fig. 10).

From the continuous nature of the curves it appears likely that the action of serum is essentially the same at all concentrations of trypsin, varying in degree only, and that therefore the apparently permanent inhibition of weak trypsin is not due to destruction of the trypsin, any more than is the temporary inhibition of stronger trypsin.

(b) *The work of Hedin.* Hedin [1906, 1] found that (1) a small amount of serum had a relatively greater effect on trypsin than a large amount; (2) it was impossible completely to neutralise a given solution of trypsin by adding any amount of serum. This result is confirmed by Young [1918] whose results are illustrated in Fig. 11: this curve shows that excess of serum has no effect on the residual activity of trypsin.

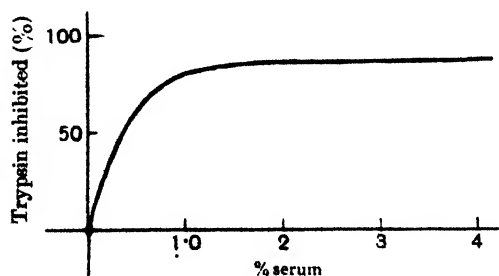


Fig. 11. Relationship between serum and inhibited trypsin [experiment by Young, 1918, p. 503, sheep]. This illustrates: (1) that degree of inhibition is a logarithmic function of amount of inhibiting serum (thus suggesting that inhibition is an adsorption phenomenon); (2) inability of serum to achieve 100 % inhibition of trypsin, the reasons for which are discussed in the text.

Hedin thus shows that a mixture of trypsin and excess of serum possesses both antitryptic and tryptic actions, *i.e.* trypsin and antitrypsin can co-exist when the latter is in excess, each exerting an independent action on added substrate or enzyme respectively.

I have not been able to confirm the common findings of Hedin and Young that excess of serum will not entirely inhibit a given amount of trypsin. Fig. 10 shows that 4 % and 2 % trypsin were completely inhibited by 10 % of serum. The cause of the discrepancy is difficult to explain, but may be due to the difference in the methods employed in studying the kinetics of trypsin. Hedin and Young combined fairly concentrated trypsin in constant amount with increasing amounts of serum: hence all flasks contained a fairly active peptidase together with a large excess of caseinogen added as substrate. Caseinogen it is stated by Jobling and Petersen [1914, 4, p. 152] is an exception to the rule that peptidase does not split proteins; furthermore, peptidase is uninfluenced by the antiferment (Jobling).

(c) *Analogy with charcoal.* To Hedin [1906, 2] is also due the discovery that charcoal inhibits trypsin in a manner kinetically very similar to that of serum. Thus the amount of trypsin bound by charcoal follows the same law as the binding of trypsin by serum: small amounts of charcoal bind relatively more trypsin than larger amounts.

This relationship, which can be expressed by the equation $x = ky^n$ (where x = amount of trypsin bound, and y = amount of active charcoal), is a logarithmic one, and is readily accounted for by assuming that the mode of action of charcoal is one of adsorption of the trypsin. Hence Hedin on the basis of kinetic similarity concluded that serum also inhibited trypsin by the process of adsorption.

The behaviour of charcoal throws some further light on the adsorptive process. Hedin showed that although a trypsin solution filtered after treatment with excess of charcoal was quite inactive, yet an unfiltered trypsin-charcoal mixture incubated with caseinogen was definitely and invariably active, though most of the trypsin remained inhibited. Hedin concluded that in the presence of subsequently added substrate charcoal did not retain all the trypsin adsorbed, a small portion of it being taken up by the substrate. In a similar manner might be explained the impossibility of totally inhibiting a solution of trypsin by means of serum, although it is impossible actually to demonstrate the similarity since trypsin cannot be removed with its inhibitor from a trypsin-serum mixture in the simple manner possible with charcoal.

(d) *Robertson's formula.* Robertson [1918] using the refractometric method, examined quantitatively the inhibition of trypsin by serum and arrived at the formula

$$\frac{T}{A(1-T)} = C$$

where T = fraction of trypsin inhibited;

A = concentration of serum present;

C = a constant representing the antitryptic value of the serum used.

Robertson claimed that his constant was an absolute measure of the inhibiting power of serum, being independent of the concentration of serum used. An examination of his results, however, shows that the fraction $\frac{T}{A(1-T)}$ tends to be constant only for mixtures containing more concentrated serum (10 % to 33 %) and even then the variation in C is too considerable (5.87 to 6.95 for a single serum) to permit of the value of C being used as a characteristic figure for the inhibiting power of serum.

Where approximate results only are required, however, the constant is a useful expression of antitryptic power, and sufficiently accurate, provided the concentration of serum in the mixture is not too small. By this method Hanson [1918] was able to show that injections of trypsin had no influence on the globulin/albumin ratio of serum, although the antitryptic index (as measured by C) rose to three times the normal value.

(e) *The specificity of the inhibiting action of serum.* Von Eisler [1905] found that human serum did not inhibit human pancreatic trypsin any more than it inhibited pig trypsin, while Weil [1910] found that serum actively inhibited the vegetable protease papain. Serum, it follows, does not exert any biologically specific action on trypsin, but inhibits all proteases alike. Apparent specificity may be shown by serum nevertheless, as the work of Young [1918] proves. He found that whereas dilute serum equally inhibited two samples of equally active trypsin obtained from the same source, the inhibition was different when the concentration of serum was increased. Fig. 12 illustrates

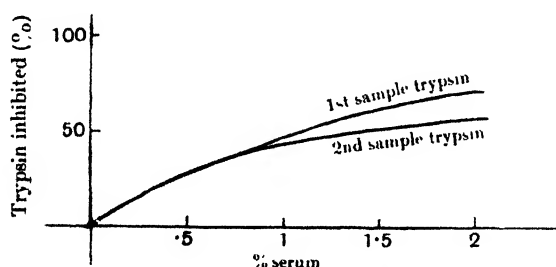


Fig. 12. Inhibition of equally active samples of ox pancreas [experiment by Young, 1918, p. 506, Exp. IV]. This shows that samples of trypsin equally inhibited by weak serum may not be equally inhibited by stronger serum.

this result, and Fig. 13 shows the result of another of Young's experiments in which equally active samples of fresh and commercial trypsin are compared. Young was unable to account for the preferential action shown by serum for certain trypsins, though this preference was not shown in all the experiments carried out: he did show, however, that the presence of zymoids could not account for the phenomenon.

His findings are important in showing the danger of attributing all variation in antitryptic action to the serum alone without first determining the uniformity of the trypsin solutions used.

The influence on serum of injections of trypsin.

A careful examination of the properties of serum-antitrypsin and of the various methods employed in measuring its value has led me to the conclusion that the failure of workers to obtain a rise in antitryptic value of serum following injections of trypsin could adequately be accounted for by one or more of the following circumstances¹.

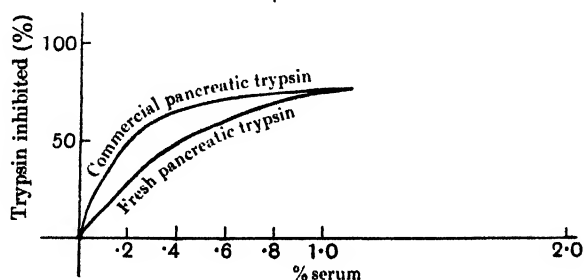


Fig. 13. Inhibition of equally active samples of fresh and commercially prepared trypsin [experiment by Young, 1918, p. 505, Exp. 1]. Here weak serum inhibits the two samples differently, the difference diminishing as the serum becomes stronger.

(a) The antitryptic power of serum is not constant in value, but follows a swinging course which in the normal animal is often determined by definite physiological conditions. Thus Jobling *et al.* [1915, 1] found a definite rise a few hours after a meal, a result which I have confirmed.

(b) It is generally agreed that the variations in antitryptic value are not very great. In Hanson's experiments [1918] the rise never exceeded three times the normal index. Consequently if the serum were antitryptically at its peak, an injection of trypsin would fail to produce a further rise in index.

(c) Hanson records that when the index returns to normal after a rise due to a trypsin injection, further repeated injections fail to produce a second rise. It is probable that such a refractory condition of serum might arise from physiological conditions other than the results of trypsin injections.

(d) The choice of method has a very important effect on the results obtained [Fine, 1930].

Experimental demonstration of the rise in antitryptic index following injections of trypsin. The results of experiments carried out with three guinea-pigs are graphically represented in Figs. 14, 15 and 16.

The method of obtaining the antitryptic index may be considered open to criticism since, in the technique employed, the substrate used is the serum itself and must therefore vary with each specimen taken: secondly, it may be objected that since substrate is not present in excess, the extent of hydrolysis

¹ Among those who failed were Doblin [1909], Rosenthal [1910], Weil [1910], Young [1918], and Pozerski [1909] (using papain instead of trypsin). Positive results, on the other hand, were obtained by Achalmé [1901], von Berman and Bamberg [1908], Jochman and Kantarovitch [1908], Meyer [1909], Jobling, Petersen and Eggstein [1915, 2] and Hanson [1918].

in 24 hours cannot be considered as representing the activity of the enzyme present.

I would answer these objections as follows.

(a) The index is calculated from the ratio of digestion of a given amount of serum-protein to the digestion of the same amount but in the absence of

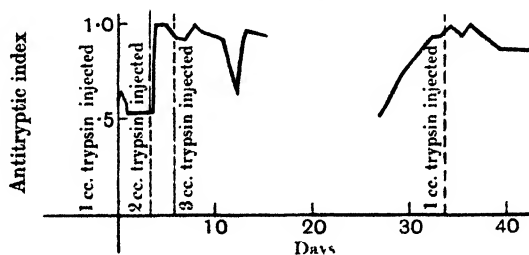


Fig. 14. Influence of trypsin injections on antitryptic index of serum. Guinea-pig *T*. This shows a rise in index immediately or shortly after each injection of trypsin. The guinea-pig was bled daily without definite relationship to the time of feeding, this leading to apparently spontaneous fluctuations in the curve.

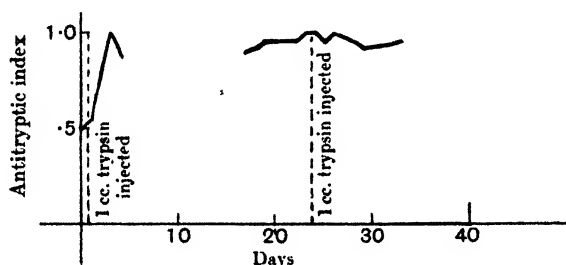


Fig. 15. Influence of trypsin injections on antitryptic index of serum. Guinea-pig *U*. After the first injection the index rises and remains high throughout the experiment.

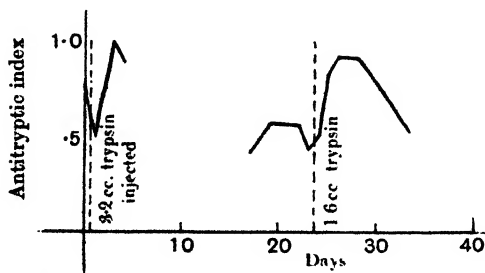


Fig. 16. Influence of trypsin injections on antitryptic index of serum. Guinea-pig *V*. Both injections are definitely associated with a subsequent rise in index. The very large first injection produced a preliminary fall.

any inhibition. Actually of course the index is the difference between this ratio and unity. Hence the actual amount of protein present is not of consequence provided it does not vary greatly from specimen to specimen: in practice the variation was not found to be great.

(b) It is true that the acidity measured is not a true measure of the activity of enzyme present: it must be realised, however, that in the method used it is not the activity of the inhibited enzyme that is measured, but the extent to which it has in a given time recovered from the inhibiting action, this degree of recovery being directly related to the degree of inhibition.

An important advantage of the method used is the possibility of distinguishing between the free acid and "formol" acid produced: since the formation of free acid only is opposed by serum, the index is in all cases calculated with reference to free acid formation only.

The three guinea-pigs experimented on were kept under observation for over a month: in each case there was a gap of 14 days during which, as can be seen, no examinations were made owing to an interruption in the investigation.

Guinea-pig *T* did not respond to any extent to the first injection of 1 cc. of trypsin (Benger's), but showed a rise within a few hours of a second injection of 2 cc., which persisted for some time. After a month the index was swinging considerably, with a tendency to rise, which a third injection of 1 cc. maintained. Guinea-pig *U* demonstrated in unequivocal fashion the rise in index in response to an injection of 1 cc. trypsin. The rise was maintained even after 20 days, a second injection having no observable effect. Guinea-pig *V* also responded to a first injection, although the rise was here preceded by a definite fall in index, accompanied by symptoms of collapse (immobility of many hours' duration). In 17 days the index had returned to a lower level, and again responded to an injection (of half the first dose): the rise was maintained for a week. A fall in index immediately following a large injection of trypsin seemed to be associated with symptoms of collapse. Thus guinea-pig *Q* became immobile, following an injection of 2.5 cc. of trypsin, and, although recovery set in in 20 minutes, the index fell from 0.6 to 0.4 in 5 hours, and death ensued in 22 hours. In this case the trypsin was not neutralised, though in all other cases it was brought to a p_H of 8.3 before injection: the route in all cases was intraperitoneal. It will be seen that although guinea-pigs *T*, *U* and *V* responded similarly to adequate injections of trypsin, they differed in their subsequent condition, the index of *T* tending to remain high, but swinging, that of *U* remaining persistently high, while that of *V* tended to return to normal in a few days.

The nature of serum-antitrypsin.

There are two questions which it is necessary to answer if the nature of serum-antitrypsin is to be known: firstly, what is the substance in serum responsible for the inhibition of trypsin? secondly, what is the mode of action of this substance on trypsin?

(1) *The antitryptic substance.* The three main theories as to identification of the antitryptic substance are described below, and their relative merits then discussed: the antibody theory is treated as a variation of the protein theory.

(a) *Amino-acid theory.* Bayliss first showed [1904] that the end-products of tryptic action retard the action of trypsin: Abderhalden and Gigon [1907] confirmed the fact that trypsin was retarded by amino-acids. On the strength of such observations Rosenthal [1910] suggested that serum-antitrypsin owed its action to the amino-acid content of serum. Walters [1912] pointed out that the action of amino-acids is too slight to account for the behaviour of serum. More recently Hussey and Northrop [1923] suggested that the polypeptides formed by tryptic action are the source of serum-inhibition. No serious proof has been offered for either of these theories, and the occurrence of the Dastre phenomenon contradicts them emphatically: end-products of tryptic action cannot reasonably account for an antitryptic action which is at its maximum at the commencement and progressively diminishes.

(b) *Lipoid theory.* Schwartz first showed [1909] that extraction with ether lessened the antitryptic power of serum, and considered this as proof that serum owed its antitryptic action to its lipoid content. His findings were confirmed by Sugimoto [1913], and the lipoid theory in a modified form was strongly urged by Jobling and his colleagues in a series of papers entitled "Studies in ferment action" [1914, 1-4]. Jobling submitted evidence showing (1) that unsaturated fatty acids and soaps possessed definite antitryptic action; (2) that saturated fatty acids and soaps possess no such antitryptic action; (3) that the iodination or hydroxylation of unsaturated fatty acids or soaps leads to the loss of antitryptic action: similar treatment of serum leads to the loss of antitryptic action of serum. This is confirmed by Slovzov and Zenophentova [1919]; (4) that although unsaturated soaps do not lose antitryptic action when heated alone, they do lose this action if heated at 70° for half an hour in the presence of serum; (5) that the chloroform extract of serum will exert an antitryptic action if saponified, although without this treatment the extract will not exert such action.

A more careful scrutiny of Jobling's experimental results, however, reveals the following facts: (1) On no occasion is there any record of an unsaturated lipoid exerting antitryptic action without previous saponification. (2) Insufficient regard is paid to the p_H of the enzyme mixtures. (3) The attempt is made to bridge the gap between antitryptic lipoids and serum as regards the effect of heat by showing that unsaturated soaps do lose antitryptic power when heated in the presence of serum. From Jobling's figures, however, it is clear that loss of antitryptic power of the unsaturated soap takes place on mixing with serum in the cold, and that heat merely increases the loss: acidity of the soap-serum mixture also increased the loss.

That the lipoids of serum have any connection with the antitryptic action of the latter is contradicted by the work of Cobliner [1910], who found that dry serum retains its antitryptic action after extraction with chloroform or other fat solvents.

Finally, I have shown that the nature of the inhibition produced by serum is essentially different from that produced by unsaturated soaps. The initial

effect of 0.5 % of sodium oleate is to stimulate trypsin, which after 24 hours shows lessening of activity. Serum, on the other hand, initially exerts its maximum inhibition on trypsin, which may ultimately recover completely. This contrast is illustrated in Fig. 17 which represents one of several experiments carried out.

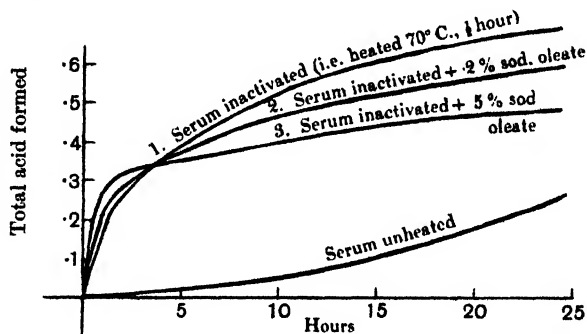


Fig. 17. Comparison between inhibition of trypsin by unsaturated soap and inhibition by serum. This shows that sodium oleate initially stimulates and ultimately depresses trypsin, whereas serum exerts an initial inhibition from which trypsin progressively recovers.

These curves were obtained by plotting the course of digestion of the following four tubes, the usual Sørensen's technique being followed.

	Tube 1	Tube 2	Tube 3	Tube 4
Serum	0.2	0.2	0.2	0.2
		inactivated	inactivated	inactivated
Trypsin 50 % (neutralised)	0.4	0.4	0.4	0.4
Buffer (p_H 8)	0.9	0.9	0.9	0.9
Sodium oleate 2 %	0.0	0.5	0.2	0.0
Distilled water	0.5	0.0	0.3	0.5

Figures represent cc.

(c) *The protein theory.* It has been definitely shown that the antitryptic action of serum is associated with the protein fraction [Landsteiner, 1900; Cathcart, 1904; and others], the albumin fraction being more antitryptic than the globulin.

Fujimoto [1918] obtained crystallised albumin and found it to be highly antitryptic. He admitted the possibility of some other constituent of serum entering into the composition of the crystals however, and on the whole considered that the antitryptic action was due to the serum-proteins and possibly some other undetermined factors in addition. Oppenheimer [1913] came to the similar conclusion that antitryptic action of serum was due to multiple causes, of which the peculiar configuration of the protein molecule was one [Oppenheimer and Aron, 1903]. Teale and Bach [1920] concluded that the nature of the serum-proteins was the cause of the antitryptic action of serum.

Beaton [1922] found however that the antitryptic index may rise without any change in the albumin content of serum, and in some cases the rise may even be accompanied by a fall in serum-albumin.

There is therefore some additional factor in the action of serum besides

the presence of protein, and this factor is in all probability a physical one, namely the degree of dispersion of the proteins.

(2) *Mode of action of proteins.* The classical work of Hedin [1906, 1, 2] confirmed by Hata [1909], Bayliss [1923], Young [1918] and others, has shown that the mechanism of inhibition by serum is one of adsorption of trypsin by the serum-proteins. Such a mechanism is perfectly reconcilable with Beaton's findings since the amount of adsorption depends not only on the amount of the adsorbing colloids, but also on their degree of dispersion. Thus the antitryptic index of serum might increase without the concentration of proteins necessarily increasing, for the existing amount of circulating protein, by an increase in its state of dispersion, can give rise to a greatly increased surface for adsorption, and therefore to a heightened capacity to adsorb trypsin. It can be shown by a simple calculation that when any particle undergoes division into n smaller particles, the total surface of the smaller particles is $\sqrt[3]{n}$ times the surface of the parent particle. Hence, if the degree of trypsin inhibition can be considered as directly proportional to the surface presented by the colloidal particles of the serum-protein, then the antitryptic index can be doubled either by doubling the amount of protein without altering the average size of colloidal particle, or by the formation of eight colloidal particles from each original one without altering the total amount of circulating protein.

The mechanism of adsorption would also serve to explain one important difference between trypsin inhibition and the inhibition of other enzymes by serum—namely the capacity of trypsin ultimately to recover from the inhibition, there being no such recovery reported for other enzymes. As Bayliss points out [1923], the adsorbed trypsin is able slowly to attack the serum-proteins adsorbing it until the latter are entirely hydrolysed and the trypsin is thus liberated.

When another enzyme such as invertase is inhibited by serum then, on the assumption that the inhibition is also due to adsorption by serum-proteins, it would not be expected that recovery should take place, since invertase would be unable to hydrolyse the adsorbing bodies. In my own experiments with invertase I found that inhibition was not recovered from; thus an invertase solution when unbuffered produced 10 % inversion of 2 % cane sugar in 24 hours and 30 % inversion in 3 days, while with a similar unbuffered solution in the presence of 10 % serum no inversion took place after 3 days.

Adsorption however is not a complete explanation of serum-antitrypsin; since all enzymes before exerting their specific action must be adsorbed by their respective substrates it still remains to be explained why trypsin should be inhibited when it is adsorbed by serum-proteins, although it acts freely when adsorbed by other protein substrates.

Discussion of the antibody theory.

There is no doubt that kinetically the action of serum on trypsin resembles that of antiserum on toxin: in both cases the amount of serum required is not proportional to the amount of substance neutralised, the ratio of serum required increasing very considerably as neutralisation of the substance is approached. The relationship is represented by a logarithmic curve such as satisfies the requirements of an adsorption phenomenon.

It may therefore be said that immunological antibodies and serum anti-trypsin resemble each other in the following respects.

(a) Both are proteins acting by means of adsorption.

(b) They can be increased in amount by injections of the substances they antagonise.

(c) Both are normally present in serum.

On the other hand the following differences between them exist.

(a) Immune antibodies are associated with the globulin of serum: anti-trypsin with the albumin chiefly.

(b) The injection of antigens can lead to increase of immune bodies to the extent of thousands of times the normal amount: whereas even an active response to trypsin injections will only treble the normal antitrypsin content.

(c) The immune antibodies are highly specific, increased capacity to neutralise a particular toxin being attended by a low neutralising power for other toxins: there is never any marked variation in the power of serum to inhibit the various enzymes normally inhibited by it.

The antibody theory of trypsin therefore amounts to little more than an expression of the fact that both antitoxic action and antitryptic action are adsorption phenomena in which the adsorbing bodies are proteins.

On the occurrence of non-amide linkages in the protein molecule.

In examining the meaning of the Dastre phenomenon, the newer knowledge of the heterogeneous nature of trypsin and erepsin was ignored in order to avoid complication of the issue: in any case it still remains true that the proteolytic enzymes can broadly be divided into two groups—one attacking chiefly the protein molecules and the larger polypeptides, and the other attacking mainly the smaller molecules.

The conclusion that serum behaved as an antiprotease was an expression of the fact that one phase only of the action of trypsin was inhibited. If the action of trypsin on a protein be examined by Sørensen's method it will be found that at the commencement free acid production predominates over formol acid, but that ultimately "formol" acid overtakes free acid.

Since the first action of trypsin is to hydrolyse the protein molecule into polypeptides, the predominance of free acid formation may be correlated with the power to attack the protein molecule, while predominance of "formol" acid may denote hydrolysis of the earlier products of activity. This correlation, which has hitherto been assumed, is borne out by the behaviour of pepsin,

the hydrolysis of proteins by which to the peptone stage only is correlated with the preponderance of free over "formol" acid throughout the period of digestion.

Strictly speaking however it cannot be assumed that free acid formation is the sole activity of the enzyme attacking the protein molecule since, as Haldane points out [1930], the specificity of the proteolytic enzymes is not absolutely fixed, each enzyme being capable of effecting a number of allied hydrolytic reactions. Consequently the inhibition of free acid formation does not necessarily prove the inhibition of protease, but denotes the suppression of one form of activity of protease.

The significance of free acid formation. Plimmer pointed out [1908] that in addition to the carbamic linkage —CONH— there may be present in the protein molecule (1) the arginine linkage —CH—NH—C— ; (2) the diketopiperazine linkage; (3) among the hydroxy-acids, the ether, ester, and anhydride linkages.

He did not consider it proved that such linkages did exist, however, and even quite recently Gränacher [1929], who believes the presence of the ester linkage highly probable in proteins, considers definite proof of this wanting.

It seems to me that the formation of free acid during hydrolysis of serum-proteins is proof of the existence of non-amide linkages. Clearly the hydrolysis of —CONH— by forming carboxyl- and amino-groups, cannot increase free acidity, but will increase "formol" acidity.

Free acid could, however, be produced by hydrolysis of esters; since this is usually the function of special enzymes (lipase and phosphatase) it is necessary to eliminate the possibility of free acid formation being due to them. In my own experiments this can readily be done.

(1) The commercial trypsin used was kept in the ice chest, and at p_{H} 2, which is destructive of lipase. Even if lipase were present in the trypsin, and were responsible for free acidity by action on serum-fats, the action would have been greater on unheated serum than on heated, since guinea-pig serum possesses a very active lipase [Fine, 1930].

(2) The action of phosphatase can be eliminated if the amount of available phosphate in serum be considered. On the basis for the figure for human serum (0.005 % phosphorus) the maximum amount of free phosphoric acid obtainable from 0.2 cc. of a 10 % solution of serum (the amount used in my titrations) is equivalent to 0.01 cc. $N/100$ NaOH—a negligible value within the range of error of the experiments.

That the function of pepsin is to hydrolyse linkages other than —CONH— is proved by the large proportion of free acid to the total acid developed during hydrolysis of proteins [Wigglesworth, 1928]. There is also more definite evidence of this function. Thus Harris showed [1923] that —SH groups were unmasked during peptic digestion, and Abderhalden and Schwab [1930] showed that pepsin completely hydrolysed dileucylthyroxine, although one of the leucine molecules was combined as an ester. Haldane suggests that in

the latter instance the ester linkage was split by a lipase present, but it is unlikely that lipase would act at the optimum p_H of pepsin.

It is very significant that in a list of 53 polypeptides, of which 29 were hydrolysed by trypsin and 24 were not, all those containing tyrosine (four), cystine (two) and *isoserine* (one), *i.e.* the only amino-acids with potentialities for other than —CONH— linkages, were in the hydrolysable group [Plimmer, 1908]. There is no mention however as to whether free acid increased during any of the hydrolyses, and therefore the nature of the linkages is not proved.

Serum antitrypsin as a resistance of the non-amide linkages of serum-proteins. If free acid formation is admitted to be the result of hydrolysis of other than —CONH— linkages, then the inhibition by serum-proteins of free acid formation must be due to a special resistance of these other linkages, a resistance which is overcome by heating at 70° for half an hour. This effect of heat is not due to coagulation, since serum heated with buffer at p_H 8 became only faintly turbid, yet lost its antitryptic action. Possibly the molecules adjoining the special linkages are stereochemically antagonistic to the action of trypsin, but are sterically altered by heat. Such stereochemical specificity is met with throughout enzyme reactions, one of the simplest examples being the resistance of glycyl-*d*-leucine, but not glycyl-*l*-leucine to hydrolysis by yeast-*erepsin* [Abderhalden and Handovsky, 1921]. That proteins can become less hydrolysable by trypsin as a result of stereochemical alteration has been shown by Dakin and Dudley [1913] who found that partial racemisation of caseinogen by alkali (the rotary power falling to 60 %) made it resistant to trypsin; Lin, Wu and Chen [1928] however found that resistance, although marked, was not complete.

But serum not only resists trypsin: it adsorbs it also, rendering it unavailable for other substrates present. The complete mechanism of serum-antitrypsin might therefore be conceived as

- (a) an adsorption, at the ester and other non-amide linkages, of those constituents of trypsin capable of attacking them;
- (b) a resistance to hydrolysis on account of neighbouring stereochemical influences;
- (c) a breakdown of the resistance, whether through stereochemical changes or other causes.

CONCLUSIONS.

1. The influence of serum on enzymes can be divided into the following categories.

(a) *Serum contains the enzyme.* Here an apparent acceleration is produced (*e.g.* diastase).

(b) *Serum accelerates an enzyme, although it does not contain it.* This is true acceleration (*e.g.* invertase at p_H 4).

(c) *Serum contains the enzyme, but accelerates it more than is accounted for by this fact* (*e.g.* lipase).

(d) *Serum inhibits the enzyme.*

2. The effect on invertase varies uniformly with p_H , at certain values of which inhibition takes place.

3. The action of serum is sufficiently accounted for by the change of p_H produced by the serum (*e.g.* pepsin) or by the adsorption of the enzyme by the serum proteins (*e.g.* trypsin), or by both factors (most other enzymes).

4. A comparison between serum-antitrypsin and a typical Ehrlich antibody such as antitoxin shows that they resemble each other in the way they act on trypsin and toxin respectively—*i.e.* by adsorbing them—and in their increase following injections into the animal body of the respective "antigens": nevertheless the increase in antitrypsin is trivial and difficult to obtain compared with the great and constant increase in antitoxin.

5. The inhibition of trypsin by serum is recovered from if digestion is continued. An analysis of the free and "formol" acid figures shows that the inhibition is directed against free acid formation, and that the initially low "formol" acid production is merely the result of shortage of substrate for the peptidase of trypsin.

6. This phenomenon of recovery rules out the lipid and the amino-acid theories of serum antitrypsin.

An examination of the evidence makes it clear that the proteins are the bodies responsible for the antitryptic action of serum, and that they act by adsorption.

7. The formation of free acid during tryptic and peptic digestion can only be the result of the hydrolysis of other than —CONH— linkages when the presence of other ester-hydrolysing enzymes is eliminated. The behaviour of serum suggests that it is at the non-amide linkages that adsorption of the inhibited enzyme takes place, while the resistance itself may be due to an unfavourable stereochemical configuration.

I have pleasure in expressing my acknowledgments to Prof. D. B. Blacklock, formerly Director of the Sir A. L. Jones Laboratory, for permission to publish details of experiments carried out in his laboratory.

REFERENCES.

- Abderhalden and Gigon (1907). *Z. physiol. Chem.* **53**, 521.
— and Handovsky (1921). *Fermentforsch.* **4**, 316.
— and Schwab (1930). *Fermentforsch.* **11**, 164.
Achalme (1901). *Ann. Inst. Pasteur*, **15**, 737.
Bayliss (1904). *Arch. Sci. Biol.* **11**, Supplement 261.
— (1912). *J. Physiol.* **43**, 455.
— (1919). *General physiology* (Longmans, London).
— (1923). *Nature of enzyme action*, chap. 8 (Longmans, London).
Beaton (1922). *Brit. J. Exp. Path.* **3**, 224.
Benzley and Harvey (1912). *Biol. Bull.* **23**, 225.
von Berman and Bamberg (1908). *Klin. Woch.* **45**, 1396.
Blacklock, Gordon and Fine (1930). *Ann. Trop. Med. and Parasitol.* **24**, 1.
Blum and Fuld (1906). *Z. klin. Med.* **58**, 505.
Buchner and Haahn (1910). *Biochem. Z.* **26**, 171.
Burge (1915). *J. Parasitol.* **1**, 179.

- Cathcart (1904). *J. Physiol.* **31**, 497.
 Cobliner (1910). *Biochem. Z.* **25**, 494.
 Czapek (1903). *Ber. deutsch. bot. Ges.* **21**, 229.
 Dakin and Dudley (1913). *J. Biol. Chem.* **16**, 505.
 Dastre (1903). *Compt. Rend. Soc. Biol.* **55**, 633.
 ——— and Stassano (1903). *Compt. Rend. Soc. Biol.* **55**, 130, 254.
 Doblin (1909). *Z. Immunol.* **1**, 650.
 Dragsted and Vaughan (1924). *Arch. Surg.* **8**, 791.
 von Eisler (1905). *Ber. Wien. Akad.* **104**, 119.
 Feldstein and Weil (1909-10). *Proc. Soc. Exp. Biol. Med.* **7**, 61.
 Fine (1930). *Biochem. J.* **24**, 1282.
 Folin and Denis (1911). *J. Biol. Chem.* **11**, 527.
 Fujimoto (1918). *J. Immunol.* **3**, 51.
 Gränacher (1929). *Handbuch der biologischen Arbeitsmethoden*: I, II, **4**, 638.
 Gross (1908). *Arch. exp. Path. Pharm.* **58**, 157.
 Hahn (1897). *Klin. Woch.* 499.
 Haldane (1930). *Enzymes* (Longmans, London).
 Hamill (1906). *J. Physiol.* **33**, 476.
 Hanson (1918). *J. Immunol.* **3**, 139.
 Harris (1923). *Proc. Roy. Soc. Lond. B* **94**, 426.
 Hata (1909). *Biochem. Z.* **17**, 156.
 Hedin (1906, 1). *Biochem. J.* **1**, 474.
 ——— (1906, 2). *Biochem. J.* **1**, 484.
 Hildebrandt (1893). *Virchow's Arch.* **184**, 325.
 Hussey and Northrop (1923). *J. Gen. Physiol.* **5**, 335.
 Jobling and Strouse (1912). *J. Exp. Med.* **16**, 3.
 ——— and Petersen (1914, 1). *J. Exp. Med.* **19**, 3.
 ——— ——— (1914, 2). *J. Exp. Med.* **19**, 4.
 ——— ——— (1914, 3). *J. Exp. Med.* **19**, 5.
 ——— ——— (1914, 4). *J. Exp. Med.* **20**, 5.
 ——— ——— and Eggstein (1915, 1). *J. Exp. Med.* **22**, 129.
 ——— ——— ——— (1915, 2). *J. Exp. Med.* **22**, 141.
 Jochman and Kantarovitch (1908). *Munch. med. Woch.* **55**, 728.
 Landsteiner (1900). *Zentr. Bakt. Par.* **1**, 27, 357.
 Langenskiöld (1907). *Arch. Int. Physiol.* **5**, 297.
 ——— (1914). *Scand. Arch. Physiol.* **31**, 1.
 Levene and Stookey (1903). *J. Med. Res.* **10**, 217.
 Lin, Wu and Chen (1928). *Chinese J. Physiol.* **2**, 131.
 Luers and Albrecht (1926). *Fermentforsch.* **8**, 52.
 Meyer (1909). *Klin. Woch.* 23.
 Morgenroth (1899). *Zentr. Bakt. Par.* **1**, 26, 349.
 Opie (1905). *J. Exp. Med.* **7**, 316.
 Oppenheimer (1913). *Die Fermente und ihre Wirkungen*, **1**, 478.
 ——— and Aron (1903). *Hofmeister's Beiträge*, **4**, 297.
 Plimmer (1908). *Chemical constitution of the proteins* (Longmans, London).
 Pozerski (1909). *Ann. Inst. Pasteur* **23**, 205.
 Robertson (1918). *J. Immunol.* **3**, 131.
 Rosenthal (1910). *Folia Serol.* **6**, 285.
 von Schoenbom (1910). *Z. Biol.* **53**, 386.
 Schwartz (1909). *Wien. klin. Woch.* **22**, 1151.
 Slovov and Zenophentova (1919). *Russ. Physiol. J.* **2**, 261.
 Sugimoto (1913). *Arch. Exp. Path. Pharm.* **74**, 14.
 Teale and Bach (1920). *Proc. Roy. Soc. Path.* **13**, 5.
 Thaysen (1915). *Biochem. J.* **9**, 110.
 Vernon (1904). *J. Physiol.* **31**, 346.
 Walters (1912). *J. Biol. Chem.* **12**, 43.
 Weil (1910). *Amer. J. Med. Sci.* **139**, 714.
 Weinland (1903). *Z. Biol.* **44**, 1.
 Wells (1925). *Chemical pathology* (Saunders, Philadelphia).
 Wigglesworth (1928). *Biochem. J.* **22**, 150.
 Young (1918). *Biochem. J.* **12**, 499.

LXXVI. THE TOTAL CARBOHYDRATE CONTENT OF ISOLATED FROG MUSCLE.

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INTRODUCTION.

THERE are two methods in general use for the determination of the total carbohydrate content of muscle. In one [Parnas and Wagner, 1914; Parnas, 1921] alcoholic and aqueous extracts of the tissue are made, glycogen being estimated in the residue and soluble carbohydrate in the extracts. In the other [Loewi, 1918; Lesser, 1920; Meyerhof, Lohmann and Meier, 1925] the whole muscle is hydrolysed in dilute acid and the glucose formed is estimated by its reducing power. Both are liable to error since it is difficult to remove non-carbohydrate reducing substances completely. The second method gives higher values than the first. Lesser considers that this is due to incomplete extraction of a dextrin. Parnas and Meyerhof *et al.* attributed it rather to the inclusion of non-carbohydrate reducing substances split off from protein during hydrolysis and not precipitated by mercuric acetate. Bissinger [1926] introduced an adaptation of the Van Slyke [1917] method in which sugar is recovered quantitatively from a copper sulphate-lime precipitate. He and Lesser [1926] found that when this technique was used for the estimation of total carbohydrate in the mouse the extraction and hydrolysis methods gave the same results. The same procedure has been applied to the estimation of alcohol-soluble carbohydrate in nerve by Holmes and Gerard [1929] and in muscle by Anderson and Macleod [1930]. Ochoa [1930] in estimating the total carbohydrate content of frog muscle later dispensed with the separation of sugar by copper-lime precipitation and relied on the complete removal of protein and non-sugar reducing substances by West, Scharles and Peterson's [1929] method. The results of the present work show that although neutral mercuric sulphate does give lower results than mercuric acetate or chloride, copper-lime precipitation will still further diminish the reducing value of the filtrates.

The present investigation is an attempt to apply a modification of Bissinger's method to the estimation of the total carbohydrate of frog muscle and to compare the results with the values found for the glycogen and alcohol-soluble carbohydrate in resting muscles, in muscles incubated in phosphate and bicarbonate buffers and in muscles poisoned with sodium monoiodoacetate.

There is some evidence that a carbohydrate intermediate in complexity between glycogen and glucose may exist in muscle. Laquer [1914, 1921] found that in a phosphate buffer solution the total yield of lactic acid exceeds the initial content of glycogen and can usually be accounted for by the lactacidogen content. Simpson and Macleod [1927] found that the breakdown of glycogen in a muscle frozen in liquid air and allowed to thaw was much more rapid than the formation of lactic acid. Anderson and Macleod [1930] have shown that on keeping after death the glycogen content of mammalian muscle remains steady, or may increase, whilst the lactic acid content increases. Lohmann [1926] has found a trisaccharide among the products of action of muscle extract on glycogen. This trisaccharide was not fermentable by yeast and did not give rise to lactic acid. More recently Barbour [1930] has isolated a trisaccharide formed as the sole product of the hydrolysis of glycogen by muscle extract at p_H 6.3. If such a trisaccharide does exist in muscle it might well escape estimation as an alcohol-soluble carbohydrate and would almost certainly be destroyed, in part at least, by the drastic alkaline hydrolysis required for the glycogen estimation.

The method employed in this work should include such a compound, whilst non-carbohydrate reducing substances should be largely, if not entirely, excluded. It was hoped that the solutions made from the copper-lime precipitates would be free from nitrogen, but this was not found to be the case. It is however unlikely that the nitrogen-containing substances are responsible for more than a small part of the reducing value, since after incubation in a phosphate buffer the carbohydrate content of a muscle falls to about 10 % of the initial value, whilst the nitrogen content of the copper-lime precipitates does not alter appreciably, whence it may be assumed that non-carbohydrate reducing substances have not altered in amount. The error from this cause, if the whole of the final reducing value is due to nitrogen-containing substances, will not be above 10 %. Another source of error, acting in the opposite sense, is the incomplete hydrolysis of carbohydrate. Glycogen is hydrolysed under optimum conditions to the extent of 94 % and other carbohydrate, should any be present, is unlikely to give values as high as 100 %; all values in this paper are given as glucose found, without arbitrary correction. In the time allowed all the hexosephosphate will not be hydrolysed, but as the amount present in muscle is small compared with the total carbohydrate, the error from this cause will be insignificant [Meyerhof, 1930]. In some cases, such as in muscles poisoned with monoiodoacetate, where exceptionally large amounts of hexosephosphate are known to be present, special allowance must be made.

The chief difference between the results found in this work and those of previous workers is the much greater difference between the total carbohydrate content and the sum of the glycogen and alcohol-soluble carbohydrate. Glycogen values vary considerably and it is difficult to compare results found by different workers, but the alcohol-soluble carbohydrate values are much lower.

Meyerhof [1920] found as an average value for resting frog muscle, 194 mg./100 g., whereas the average resting value for the present results is 40 mg./100 g. This may be due both to the use of copper-lime precipitation and to the use of absolute alcohol as an extractant in place of 60 % alcohol, which must extract some glycogen as well as other carbohydrate and so make the results for alcohol-soluble carbohydrate too high. The values for total carbohydrate are decidedly higher than those found by other workers, although lower values would have been expected since sugar was separated by copper-lime precipitation and protein was removed after 30 minutes' hydrolysis, the total period of hydrolysis (1.5 hours) being much shorter than that usually allowed. In one experiment the values for total carbohydrate estimated by several variations of the method were compared. When hydrolysis was discontinued at the end of 30 minutes, protein removed by Schenk's method, and the hydrolysis continued for another hour, the total carbohydrate content of the muscle was 0.973 %, if sugar were separated by copper-lime precipitation, but 1.219 % if calculated from the reducing value of the solution before copper-lime precipitation. If protein were removed by West, Scharles and Peterson's method the values were 0.840 % and 1.022 % respectively, the discrepancy being less after copper-lime precipitation. When hydrolysis was continued for 3 hours before removal of protein by Schenk's method the value was 1.071 % after copper-lime precipitation and 1.347 % before; if protein were removed by West, Scharles and Peterson's method the values were 1.202 % and 1.044 % respectively, higher in all cases when protein was not removed before the completion of hydrolysis. The Schenk method was used throughout this work, as when most of the experiments were carried out the superiority of the West, Scharles and Peterson method was not appreciated; the results are therefore probably too high by 10 % to 15 %, a figure comparable with that estimated for the error due to non-carbohydrate reducing substances.

METHODS.

Preparation of material. In all the experiments recorded in this paper single isolated frog muscles were used. When working with single muscles it is essential that all comparisons should be made either in simultaneous determinations on the same muscle, or on pairs of corresponding muscles from the two legs of the same frog. The glycogen and total carbohydrate contents of resting muscles from the same frog vary very much, and a totally wrong idea of the changes taking place may result from comparing different muscles or even by taking average figures. The glycogen and total carbohydrate contents of corresponding muscles of a pair from opposite legs of the same frog agree reasonably well (Table VI), and a much truer picture of changes taking place results from confining all the estimations to one pair of muscles.

The frogs were in all cases the large Hungarian variety. They were killed by a cut across the base of the skull, followed immediately by section of the

spine just above the pelvic girdle. The muscles were dissected, weighed on a torsion balance and dropped into Ringer solution at p_H 7.3, containing 0.012 % sodium dihydrogen phosphate and 0.057 % disodium hydrogen phosphate, but no bicarbonate. Oxygen was slowly bubbled through the solution. The muscles were left in the solution for at least 1 hour and usually longer before further treatment. Dulière and Horton [1929] have shown that muscles treated in this way are not subject to the onset of inexcitability, and that their resting phosphagen content is high and inorganic phosphate low. The lactic acid content is also low, on an average 20 mg./100 g., as is shown by the results in this paper. These figures indicate that the muscles are in a resting condition and have recovered from any stimulation or injury inflicted during killing and dissection. This method is suitable only for thin muscles, such as sartorii or semi-membranosi, and cannot be used for thick muscles such as gastrocnemii, as the oxygenated solution will not diffuse into them; where such muscles were used they were treated immediately after dissection without any soaking in Ringer solution.

Estimation of total carbohydrate: extraction and hydrolysis. 2.2 % hydrochloric acid has been found most suitable for the hydrolysis of glycogen and, since glycogen forms the greater part of the carbohydrate of muscle, this strength of acid was used for the hydrolysis of the whole muscle. In the first experiments the muscles were thrown into boiling acid, but later they were ground in ice-cold acid, and extract and residue were hydrolysed together in a boiling water-bath, as changes due to killing are better avoided in this way. The completeness of the extraction of carbohydrate was tested by determining the amount of glycogen in the residue after varying the times of hydrolysis. Protein was precipitated by Schenk's method, the solid residue removed by centrifuging, and glycogen estimated in it. It was found that after 15 minutes most of the glycogen was removed, and that heating for longer than 30 minutes did not remove appreciably greater quantities, so the latter period was adopted as standard. There was always some glycogen left in the residue, and it did not change in amount during the various treatments applied to the muscle.

Hydrolysis for 30 minutes will not hydrolyse all the carbohydrate present in the muscle completely, but it is inadvisable to heat longer, owing to the danger of further protein breakdown. The time necessary for maximum hydrolysis, after removal of protein, was investigated by hydrolysing the protein-free extract, obtained from a number of muscles, in a boiling water-bath and at intervals removing portions for sugar estimation, after precipitation with copper-lime. The results are plotted in Fig. 1. In one experiment (curve b_2), the nitrogen values of the copper-lime precipitates were determined, and it will be seen that they fall off slowly, whilst the sugar values rise sharply to a peak and then fall away. This may be explained by supposing that a part of the reducing value is due to some nitrogen-containing substance which is slowly being destroyed, as is indicated by the decreasing amounts of nitrogen associated with the copper-lime precipitates. In this experiment (curve b_1), protein was

removed by trichloroacetic acid, which may make the sugar values low; although most of the acid is destroyed during the hydrolysis, sufficient is left to interfere with the sugar estimation, and for this reason the method was not used in later work. One hour was adopted as the standard period for the second hydrolysis. The curves plotted in Fig. 1, which are typical of several obtained, show that hydrolysis was complete in this time.

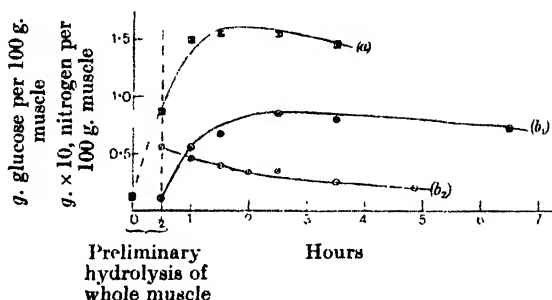


Fig. 1. Sugar formed on hydrolysis of hydrochloric acid extract of muscle.

Exp. (a). Sugar formed: protein removed by Schenk's method.

Exp. (b) 1. " " " " " " trichloroacetic acid.

(b) 2. Nitrogen content of copper-lime precipitate.

Estimation of sugar in hydrolysed solutions. Sugar was estimated in the solutions after hydrolysis by an adaptation of Bissinger's [1926] method. A further investigation of the conditions of precipitation and recovery of sugar was undertaken, using pure glucose solutions.

Freedom of precipitate from non-carbohydrate reducing substances. Holmes and Gerard [1929] state that creatinine is not precipitated by the copper-lime reagent. In order to test this the effect of treating a solution containing 25 mg./100 cc. of creatinine with copper sulphate and lime was determined. After removing the precipitate the creatinine content was reduced to 80 % of the original. That this was partly due to adsorption or occlusion on the precipitate was shown by dissolving the latter in normal hydrochloric acid and reprecipitating the copper compound with an equal volume of normal sodium hydroxide. By using two precipitations in this way it was possible to recover 95 % of creatinine in the mother-liquor.

In an experiment using a solution formed by the hydrolysis of muscle the nitrogen content of the first copper precipitate was 150 mg./100 g. of muscle. This solution gave a biuret reaction. Solution of the precipitate in normal hydrochloric acid and reprecipitation with normal sodium hydroxide reduced the value to 40 mg./100 g. muscle. This second solution gave no biuret reaction. From these results it was decided to use this method of two precipitations throughout.

Optimum conditions for precipitation of sugar. The solutions were allowed to stand for 30 minutes [Van Slyke, 1917] after addition of copper sulphate and lime, and again for 30 minutes after the second precipitation with sodium

hydroxide. If the alkaline solutions are allowed to stand too long some of the sugar will be destroyed (Table I), in fact some is destroyed in any case, for higher values are obtained when hydrochloric acid is added before copper sulphate and lime, and precipitation is not allowed to take place, the rest of the determination being carried out in the same way. The loss is a constant percentage of the whole and can be corrected.

Table I.

Effect of time of standing on recovery of sugar from copper-lime precipitates.
An equal time was allowed after each precipitation.

Time in minutes	Sugar recovered, mg.
25	5.88
30	6.16
35	6.06
40	5.94
45	5.78

The proportion of sugar recovered was constant at any one temperature, and did not vary much with temperature so long as the other conditions remained unaltered (Table II). The solutions were allowed to stand at laboratory temperature, and the factor required was redetermined from time to time, especially if there was known to be any large variation in temperature.

Table II.

Effect of variation of temperature during standing on recovery of sugar
from copper-lime precipitates.

Temperature	Sugar recovered, mg.
0°	8.9
18°	8.5
24°	7.9

Proportions of copper sulphate and lime required for complete precipitation of sugar. The amounts both of copper sulphate and of lime used by different workers vary very much. The minimum amounts required were determined for concentrations of sugar up to 0.1 % in a volume of 25 cc. The sugar remaining in solution after addition of increasing amounts of a 10 % solution of crystalline copper sulphate and a 10 % suspension of calcium hydroxide was determined, the precipitated copper compound and excess of lime being removed by centrifuging. The amount of calcium hydroxide was adjusted so that there should always be an excess present after allowing for the formation of calcium sulphate and saturation of the solution with calcium hydroxide. The amount of sugar remaining in solution decreased with increasing amounts of copper sulphate up to 2 cc., but beyond this there was no improvement, the solution after centrifuging had always about 1 % of the original reducing value. Using 2 cc. of 10 % copper sulphate, 0.94 cc. of a 10 % suspension of calcium hydroxide is required for formation of calcium sulphate and for saturation of 25 cc. of solution with calcium hydroxide; in order that there should always be a considerable excess present, 2 cc. were added.

Recovery and estimation of sugar. If a method could be found in which the copper used in precipitating the sugar could be used as the oxidising agent it would simplify the procedure very much. Folin's method [1926] seemed the most likely to be satisfactory, especially as Eggleton (private communication) has shown that it is possible to estimate the molybdenum blue, formed on addition of the acid reagent, by titration with potassium permanganate, instead of colorimetrically. Eggleton found that 1 mg. of glucose required 8.20 cc. of 0.004 *N* permanganate. This method was found very satisfactory when a modification of the alkaline tartrate reagent was made. The large excess of copper did not affect the reaction, and the final titration was sharp enough to make the accuracy of the method sufficient for work on isolated muscles.

The copper precipitate did not dissolve in the tartrate reagent, made up without copper sulphate, owing to the calcium hydroxide still present, but dissolved easily in a slight excess of normal hydrochloric acid, and an aliquot part of this solution was used for sugar estimation. It was not possible to use more than 2 cc. per estimation as Folin's special tubes were used, and as the volume of the solution after dissolving the copper precipitate in *N* hydrochloric acid was not far short of 10 cc., only about $\frac{1}{3}$ th could be taken for each determination. The tartrate reagent was made up without copper sulphate and with four times the amount of tartrate used by Folin, since $\frac{1}{3}$ th of the dissolved copper precipitate contained four times as much copper as 2 cc. of Folin's reagent. The amount of sodium carbonate was increased from 0.7 % to 7.5 % to allow for neutralisation of hydrochloric acid, the formation of calcium carbonate and for the absorption of the additional carbon dioxide liberated. The amount of sodium bicarbonate was not altered from the 2.0 % in Folin's solution. The ratio of carbonate and bicarbonate formed in this solution was found adequate for buffering changes in p_H due to variation in excess of hydrochloric acid. The accuracy of the estimation was not affected by small excess either of copper or of tartrate, nor did the calcium carbonate precipitated when the two solutions are mixed interfere with the reaction as long as the mixing was efficient.

The final composition of the alkaline tartrate solution using $\frac{1}{3}$ th of the dissolved copper precipitate for each determination was as follows:

Sodium potassium tartrate	6 %
Sodium carbonate	7.5 %
Sodium bicarbonate	2 %.

The acid solution was that recommended by Folin:

Sodium molybdate	150 g.	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	per litre
Phosphoric acid	225 cc.	85 % acid	..
Sulphuric acid	150 cc.	25 %
Acetic acid	75 cc.	99 %

Summary of procedure for estimation of total carbohydrate. The muscle, weighing usually 0.15–0.35 g., was ground with sand and 10–15 cc. of ice-

cold 2.2 % hydrochloric acid, and the mixture was hydrolysed for 30 minutes in a boiling water-bath. After cooling an excess of solid mercuric chloride was added, the solution was made up to 25 cc. and allowed to stand. It was then filtered, moist hydrogen sulphide was passed through the filtrate, the mercuric sulphide was filtered off, and the filtrate thoroughly aerated to remove hydrogen sulphide. 15 cc. were hydrolysed for 30 minutes, and after cooling the solution was washed into a centrifuge tube graduated at 25 cc. and at 10 cc.; 2 cc. of a 10 % solution of crystalline copper sulphate were added, and the solution was treated with 40 % sodium hydroxide until the copper was just precipitated. 2 cc. of a 10 % suspension of calcium hydroxide were then added, the solution was well stirred, and water was added to 25 cc. The tube was left to stand for 30 minutes and centrifuged for 3 minutes. The supernatant liquid was poured off and used for lactic acid estimation. The precipitate was dissolved in 5 cc. of *N* hydrochloric acid, 5 cc. of *N* sodium hydroxide were added, the solution was well stirred, and the volume brought back to 25 cc. After 30 minutes the precipitate was separated by centrifuging and dissolved in 3 cc. of hydrochloric acid. Less acid was needed for the second solution as the second precipitate contained less calcium hydroxide. The volume was made up to 10 cc., and 2 cc. of this solution were added to 2 cc. of the tartrate solution in a Folin tube. The sugar solution should be run into the bottom of the tartrate solution, otherwise the calcium carbonate formed greatly impedes the mixing which is absolutely essential for satisfactory results. The tube was placed in a boiling water-bath for 8 minutes, cooled for at least 1 minute in cold water, and the acid reagent was added. When all the cuprous oxide and the calcium carbonate were dissolved, the solution was washed into a large boiling-tube and titrated with 0.004 *N* potassium permanganate. The solution may safely be left for about 30 minutes after addition of the acid reagent, but should be titrated immediately after it has been washed into the tubes for titration. The end-point is not difficult to see after some practice and in a good light, but the titration must be rapid as the blue colour reappears on standing.

When the estimation was carried out in this way, the relationship between the amount of potassium permanganate required and the amount of glucose in solution was linear. The results of estimating pure glucose by this method are plotted in Fig. 2. Curve (a) was obtained using only the precipitation and sugar recovery part of the method, curve (b) by including the Schenk protein removal technique, starting with addition of hydrochloric acid and mercuric chloride. The mean value for the factor for converting cc. of potassium permanganate into mg. of glucose is 0.1 when the full method is used, but 0.094 when the Schenk procedure is omitted (temperature 16°). Possibly some glucose is lost during aeration of the solution in removing hydrogen sulphide. The recovery of added glucose in a determination of total carbohydrate in muscle was measured and the results calculated, using curve (b) Fig. 2, are shown in Table III. The accuracy of the sugar recovery is not nearly as great as in the determinations on pure glucose solutions. The inaccuracies are

probably due to conditions of extraction and hydrolysis of carbohydrate and not to errors in the sugar method.

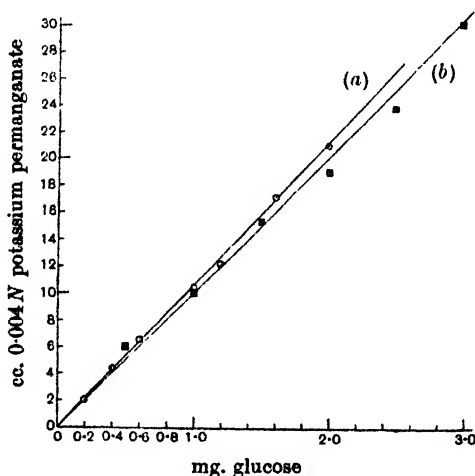


Fig. 2. Titration of glucose recovered from copper-lime precipitate against 0.004 N potassium permanganate.

(a) Recovery from copper precipitates. (b) Including Schenk protein precipitation.

Table III.

3 g. muscle extracted with 100 cc. 2.2 % hydrochloric acid, after 30 minutes' hydrolysis extract filtered and a solution containing 1, 2 and 3 mg. sugar added to portions of 10 cc. Total carbohydrate estimated in each portion.

Sugar added mg.	Carbohydrate found mg. glucose	Glucose recovered mg.	% recovery
0	1.41 1.45 Mean 1.43	—	—
1	2.62 2.39 „ 2.51	1.08	108
2	3.40 3.26 „ 3.33	1.90	95
3	4.53 4.47 „ 4.50	3.07	102

If the reducing value finally measured is due only to glucose, it should be the same when measured by different methods. In order to check this, in an experiment on muscle the reducing value of the second copper precipitate, dissolved in hydrochloric acid, was estimated by Hagedorn and Jensen's method, after removal of copper and calcium [Holmes and Gerard, 1929], as well as by this adaptation of Folin's method. When the results were corrected by a factor found by carrying out the estimations on pure glucose solutions, and necessitated by losses occurring during the copper precipitation, they agreed very well with those found by the Folin method.

Estimation of glycogen. It was necessary to estimate both glycogen and lactic acid in the same muscle, so the muscles were crushed in 10–15 cc. of ice-cold absolute alcohol in well-cooled mortars and washed into cooled centri-

fuge tubes. The tubes were left overnight in the refrigerator to allow complete extraction of lactic acid. The alcoholic extract was separated by centrifuging, the residue was washed twice with absolute alcohol, and glycogen was estimated in it as described in a previous paper [Kerly 1930], the correction for solubility in aqueous alcohol being applied. Sugar was estimated after hydrolysis by the original Hagedorn and Jensen method, by Hanes's [1929] modification or by the Shaffer-Hartmann method. Loss of glycogen is insignificant except perhaps for muscles containing very little glycogen, since the volume of alcohol is very large in relation to the volume of the muscle, and the water content of the muscle has an inappreciable effect on the concentration of the alcohol. Anderson and Macleod [1930] find that for mammalian muscle this procedure is not satisfactory for glycogen estimation, but the method is not quite comparable for the two types of muscle. In the case of mammalian muscle the muscles were cut off the bones and finely minced under alcohol, a process that must take an appreciable time, whilst the frog muscles were allowed to recover after dissection and then dropped into the ice-cold alcohol; within a few seconds at most they were completely disintegrated.

Estimation of soluble carbohydrate. Alcohol-soluble carbohydrate was estimated in the alcoholic extracts of those muscles used for estimation of glycogen and lactic acid. In some cases determinations were made of the initial reducing value only after copper-lime precipitation, in others of the reducing values both initially and after hydrolysis. For convenience the former is described arbitrarily as the alcohol-soluble sugar, the latter as alcohol-soluble carbohydrate. The alcoholic extract and washings were evaporated nearly to dryness, water was added and a few drops of benzene, and heating was continued to remove the last traces of alcohol [Boyland, 1928]; the solution was then made up to 25 cc. 15 cc. were used for determination of sugar and 10 cc. for carbohydrate. Copper sulphate and lime were added and sugar was estimated as described above. For alcohol-soluble carbohydrate hydrochloric acid was added to make 2.2 % and the solution was hydrolysed for 3 hours in a boiling water-bath, sugar being estimated in the hydrolysate. The insignificant amounts of fat and protein extracted by the absolute alcohol were not removed. The conditions chosen were those most suitable for hydrolysis of glycogen, but the time is quite inadequate for hydrolysis of hexosemonophosphate which should be included both in the determination of sugar and of carbohydrate, since it is carried down by the copper-lime precipitate [Embden and Zimmermann, 1924].

The values found for both alcohol-soluble sugar and carbohydrate were much lower than those usually quoted, probably owing to the failure of most workers to remove reducing substances other than sugar. The average values found for resting muscle were 20 mg./100 g. for sugar (of the same order as those found by Anderson and Macleod for mammalian muscle, using a copper precipitation method), and 40 mg./100 g. for carbohydrate, the variation from the mean being large in both cases. The value for alcohol-soluble sugar may be compared with that found for hexosemonophosphate by Embden and Jost

[1928] and by Eggleton and Eggleton [1929], who found a value of 5-8 mg./100 g. P corresponding with 30-40 mg./100 g. hexose. If it be assumed that hexosemonophosphate gives a reducing value corresponding to 50 % of its hexose content [Meyerhof, 1930], then 20 mg. of alcohol-soluble sugar represents 40 mg. of sugar as hexosemonophosphate. The agreement suggests that the alcohol-soluble sugar of muscle is hexosemonophosphate, but as absolute alcohol may not extract all the lower carbohydrate [Holmes, 1929] there may be other reducing sugars present. Neither Embden and Jost nor Eggleton and Eggleton used absolute alcohol as an extractant, so their results are not low for this reason. The reducing value of the alcoholic extracts after hydrolysis is approximately doubled, but hexosemonophosphate is only hydrolysed to the extent of 20 % during 3 hours in dilute acid [Meyerhof, 1930] so that there must be other carbohydrate constituents present in the alcoholic extract of muscle.

The accuracy of individual values for both alcohol-soluble sugar and carbohydrate is not high, but the values taken as a whole indicate approximately the amount present, and whether any gross changes are taking place during activity of the muscle.

Estimation of lactic acid. The method used depended on whether the muscles were used for determination of total carbohydrate or for glycogen and alcohol-soluble carbohydrate.

In a hydrochloric acid extract of muscle. When estimating total carbohydrate the lactic acid was extracted during the preliminary 30 minutes' hydrolysis. Experiments with a solution of zinc lactate in hydrochloric acid showed that none of the lactic acid was destroyed either during prolonged boiling, as long as efficient condensers were used, or during aeration to remove hydrogen sulphide. A measured volume of the supernatant fluid poured off after removal of the first copper-lime precipitate was taken for estimation of lactic acid. A modification of Friedemann, Cotonio and Shaffer's [1927] method was used, in which manganese sulphate was used to catalyse the reaction, but the aldehyde formed was distilled over in a current of steam instead of air. This method gave a mean recovery of 98 % of lactic acid from zinc lactate solutions. The recovery of lactate added to a muscle extract after hydrolysis is shown in Table IV, the results being calculated by means of the recovery factor found for pure zinc lactate solution.

Table IV.

Recovery of lactic acid from zinc lactate solution added to a hydrochloric acid extract of muscle after the first hydrolysis and before removal of protein.

Zinc lactate added mg. lactic acid	Lactic acid found, mg.	Lactic acid recovered mg.	% recovery
0	0.098	—	—
0.422	0.525	0.427	101
0.633	0.741	0.643	101
0	0.114	—	—
0.379	0.504	0.390	103
1.140	1.314	1.200	106

In an alcoholic extract of muscle. When estimating glycogen lactic acid was extracted by the alcohol, the pulverised muscles being left overnight in the refrigerator to ensure complete extraction. Experiment showed that washing with two portions of 5 cc. of absolute alcohol was sufficient to remove all the lactic acid. Lactic acid was estimated, as described above, in the mother-liquor of the first copper-lime precipitate in the portion of the alcoholic extract used for alcohol-soluble sugar estimation. The temperature at which the alcohol is removed must not rise much above 60° as if the solution becomes too hot or if it is evaporated absolutely to dryness, loss is liable to occur in the lactic acid content. In any case the recovery of added lactic acid is not so good as from a hydrochloric acid extract of muscle. The mean of a number of experiments (Table V) was 85 % recovery, and the amounts of lactic acid found were corrected by this figure. The small quantities of fat and protein, which were not removed, were found not to interfere with the estimation.

Table V.

Recovery of lactic acid from an alcoholic extract of muscle, extract divided into two equal portions and 1.03 mg. lactic acid (as zinc lactate) added to one portion, (B), lactic acid estimated as described in text in both portions.

Portion A lactic acid mg.	Portion B lactic acid mg.	Lactic acid recovered mg.	Recovery %
0.247	1.095	0.848	82
0.254	1.187	0.933	91
0.259	1.170	0.911	88
0.229	1.106	0.877	85
0.265	1.173	0.908	88
0.209	1.030	0.821	80
0.129	0.992	0.863	84
0.101	0.925	0.824	80
0.441	1.326	0.885	86
0.305	1.153	0.849	82

Mean 85

A COMPARISON OF THE CHANGES TAKING PLACE IN THE TOTAL CARBOHYDRATE CONTENT AND IN THE DIFFERENT CARBOHYDRATE FRACTIONS.

The total carbohydrate, glycogen and alcohol-soluble carbohydrate of resting muscles. The total carbohydrate content and the glycogen content of pairs of muscles from opposite legs of frogs are shown in Table VI. The muscles were all in a resting condition, having recovered from dissection in oxygenated Ringer solution. The agreement between the two muscles is not very exact, but can be said generally to fall within 10 %. The variation from one muscle to another in the same frog is considerable and is greater for total carbohydrate than for glycogen.

In order to compare the total carbohydrate content with the glycogen content one muscle of a pair was analysed for total carbohydrate, the other for glycogen. In some cases the alcohol-soluble sugar, and in two cases the alcohol-soluble carbohydrate, was also determined. In those cases where the alcohol-

Table VI.

Total carbohydrate and glycogen content of pairs of muscles from opposite legs of a frog, muscles allowed to recover in oxygenated Ringer solution.

Frog	Muscle	Weight mg.	Total carbohy- drate mg. per 100 g.	Glycogen mg. per 100 g.	Differ- ence %
I	S	319	969	—	3
		311	938	—	
I	Sm	413	1110	—	16
		408	1320	—	
I	B	276	868	—	7
		280	929	—	
II	Sm	377	607	—	2
		397	595	—	
II	B	250	460	—	10
		268	513	—	
III	B	175	920	—	9
		174	834	—	
IV	S	310	1310	—	7
		335	1220	—	
V	S	317	—	523	10
		342	—	463	
VI	B	266	—	568	1
		270	—	563	
VI	S	324	—	648	3
		325	—	668	
VII	S	335	—	758	9
		369	—	694	
VIII	B	217	—	571	6
		241	—	539	
VIII	S	305	—	495	8
		317	—	457	
*IX	G	1131	—	675	9
		1169	—	742	
*X	G	1584	—	558	4
		1588	—	578	

Note. In this and subsequent tables S=Sartorius, Sm=Semi-membranosus, B=Biceps, G=Gastrocnemius, Gr=Gracilis.

* Immediately after dissection, no recovery in Ringer solution.

Table VII.

Comparison of total carbohydrate, glycogen and alcohol-soluble sugar and carbohydrate of pairs of muscles, resting, mg. per 100 g. muscle.

Frog	Muscle	Weight mg.	Total carbo- hydrate	Glycogen	Alcohol- soluble sugar	Alcohol- soluble carbo- hydrate	Excess total carbo- hydrate over gly- cogen
I	S	315	810	591	30	(60)	219
I	Sm	371	1375	915	14	(28)	460
I	B	224	1307	735	34	(68)	570
II	S	239	965	636	—	—	329
II	B	192	995	721	—	—	274
III	S	203	758	656	49	47	102
IV	Sm	352	1775	931	52	92	844

soluble carbohydrate was not determined, an estimated value for this equal to twice the alcohol-soluble sugar content is included in Table VII for comparison, the values so calculated are enclosed in brackets.

The glycogen content is in all cases much less than the total carbohydrate content, the greatest variation is shown where the total carbohydrate is highest. In no case does the alcohol-soluble carbohydrate account for more than a fraction of the excess of total carbohydrate over glycogen. The values for alcohol-soluble carbohydrate may be low, owing to inefficient extraction from the muscle and to incomplete hydrolysis of the carbohydrates present, *e.g.* hexosephosphate, but when every allowance is made for these corrections, it is impossible that the alcohol-soluble carbohydrate can account for the difference between the glycogen and total carbohydrate. The difference also is too great and too regular to be accounted for by the variation in total carbohydrate and glycogen content of two muscles of a pair, and it is much greater than the maximum amount of non-carbohydrate reducing substance likely to be associated with the total carbohydrate fraction. It may in part be accounted for by ribose derived from adenylic acid. Ochoa [1930] states that as much as 100 mg./100 g. muscle may arise from this source. But even taking into consideration both ribose and non-carbohydrate reducing substances the difference is so great that there must almost certainly be some other substance present.

Changes taking place during incubation in phosphate and bicarbonate buffer solutions. The muscles used in these experiments were allowed to recover from dissection in Ringer solution. One muscle was then analysed and used as the resting control, the other was cut finely with a pair of scissors and placed in a test-tube under 1–2 cc. of the buffer solution. The tube was placed in an incubator at 37° for 3 hours, then the cut muscle was analysed in the same way as the control. The buffer solution was added to the extract. So that estimations of total carbohydrate should be comparable with estimations of glycogen and soluble carbohydrate both experiments were carried out on frogs from the same batch.

Phosphate buffer. A mixture containing 95 % *M*/5 disodium hydrogen phosphate and 5 % *M*/5 sodium dihydrogen phosphate was used, p_H 8. The results are shown in Tables VIII and IX. The total carbohydrate and lactic acid changes and some glycogen and lactic acid changes were estimated using autumn frogs. A more extensive set of experiments on the glycogen and lactic acid changes was carried out on two further batches, one of autumn and one of summer frogs.

In all the cases examined nearly the whole of the carbohydrate of the muscle has disappeared, a mean value of 92 mg./100 g. muscle remaining, or 5–14 % of the initial content, with an average value of 9 %. In no case is the increase of lactic acid equal to the decrease of carbohydrate, in all cases except the first a large amount of carbohydrate has disappeared that has not been converted into lactic acid. It is possible that some or all of it has been changed into hexosephosphate, but from the large difference between the carbohydrate unconverted to lactic acid and the residual value, it is not likely that the final

Table VIII.

Change in glycogen, lactic acid, alcohol-soluble sugar and carbohydrate of muscle incubated for 3 hours at 37°, mg. per 100 g.

Frog	Muscle	Weight mg.	Glycogen initial final change	Alc.-sol. sugar initial final change	Alc.-sol. carbo- hydrate initial final change	Lactic acid initial final change
(a) Phosphate buffer, autumn frogs, same batch as Table IX (a).						
I	Sm	210	749	2		10
		212	67 - 682	9 + 7	—	615 + 605
I	S	172	400	0		4
		162	72 - 338	19 + 19	—	341 + 337
II	Sm	164	916	10		10
		161	350 - 566	46 + 36	—	592 + 582
(b) Phosphate buffer, autumn frogs, another batch.						
III	B	246	710			74
		206	102 - 608	—	—	707 + 633
IV	Sm	509	1090			14
		478	60 - 1030	—	—	989 + 975
(c) Phosphate buffer, summer frogs.						
V	S	244	598			17
		—	76 - 522	—	—	700 + 683
V	B	235	569			63
		—	72 - 497	—	—	603 + 540
VI	S	274	555			18
		—	84 - 471	—	—	622 + 604
VI	B	261	605			15
		—	92 - 513	—	—	632 + 617
VII	S	293	588			14
		—	89 - 499	—	—	564 + 550
VIII	S	259	802			5
		—	97 - 705	—	—	703 + 698
IX	B	175	696			9
		—	137 - 559	—	—	559 + 550
(d) Bicarbonate buffer, autumn frogs, same batch as Table IX (b).						
X	S	198	915	24	35	44
		186	469 - 446	23 - 1	43 + 8	241 + 197
XI	Sm	181	1150	0	36	26
		192	400 - 750	42 + 42	57 + 21	695 + 669
XI	B	112	951	24	27	21
		112	594 - 357	12 - 12	72 + 45	337 + 316
XII	B	190	754	47	16	56
		189	473 - 281	58 + 11	79 + 63	444 + 388
XIII	Sm	199	1855	20	0	79
		200	799 - 1056	65 + 45	85 + 85	445 + 366

reducing value is all due to hexosephosphate. It is also possible that this final reducing value is due partly or entirely to non-carbohydrate reducing substances, since the copper-lime precipitates were not free from nitrogen. If this excess of reducing substance is converted into a compound allied to lactic acid it should be estimated, in part at least, with lactic acid, though it might possibly be destroyed during the acid hydrolysis.

The amount of carbohydrate which is changed into lactic acid is of the

Table IX.

Change in total carbohydrate, lactic acid and "residual glycogen
in muscles after incubation for 3 hours at 37°, mg./100 g.

Frog	Muscle	Weight mg.	Total carbo- hydrate	Lactic acid	Residual glycogen	
			initial final change	initial final change	(i) initial final change	(ii) initial final change
(a) Phosphate buffer, autumn frogs, same batch as Table VIII (a).						
I	Sm	215	720	24	64	87
		215	98 - 622	640 + 616	80 + 16	103 + 16
I	B	144	582	34		
		114	60 - 522	283 + 249	—	—
II	Sm	473	1610	20	28	39
		490	84 - 1526	1030 + 1010	16 - 12	32 - 7
II	B	322	1092	8	45	64
		304	119 - 973	608 + 600	79 + 34	101 + 37
III	Sm	243	1250	42	*34	*53
		256	85 - 1165	487 + 445	*35 + 1	*55 + 2
IV	B	231	1695	20	*25	*49
		231	108 - 1587	970 + 950	*32 + 7	*65 + 16
(b) Bicarbonate buffer, autumn frogs, same batch as Table VIII (d).						
V	Sm	264	1335	38		
		234	831 - 504	486 + 448	—	—
V	B	162	1055	34		
		157	658 - 397	604 + 570	—	—
VI	S	152	826	50		
		154	309 - 517	462 + 412	—	—
VII	Sm	210	1530	55		
		182	527 - 1003	793 + 738	—	—
VIII	S	161	1298	33		
		173	564 - 734	745 + 712	—	—

(i) Residual glycogen calculated without solubility correction.

(ii) Residual glycogen calculated including solubility correction.

* Shaffer-Hartmann sugar estimation.

same order as the amount which can be accounted for as glycogen when the total carbohydrate and glycogen are determined on the same muscle (Table VII). This is in agreement with the evidence of Table VIII, that the greater part of the lactic acid is formed from glycogen. These experiments do not give sufficient evidence to determine the fate of the carbohydrate not converted into lactic acid.

Laquer [1914, 1921] found that during incubation in phosphate buffer the increase in lactic acid is sometimes greater than the initial carbohydrate content, measured by adding together the glycogen and the lactacidogen. In only two of the results in Table VIII is the initial glycogen content less than the increase in lactic acid, but in several the increase of lactic acid is greater than the decrease in glycogen. This is more often the case with summer frogs than with autumn frogs, and may be a seasonal difference, but both types of result are found in both groups of frogs. The change in alcohol-soluble sugar was only measured in three cases, in all of which it increased, probably owing to the formation of hexosemonophosphate. This increase in alcohol-soluble sugar makes the difference between the decrease of the sum of glycogen and

soluble sugar, and the increase of lactic acid greater where the lactic acid change is already greater than the glycogen change, and more nearly equal, or greater, in the remaining cases, and brings the figures generally more into agreement with those of Laquer. But when the total carbohydrate is measured by acid hydrolysis, as already discussed, the position is quite different (Table IX *a*).

Determinations of residual glycogen, *i.e.* glycogen left in the solid residue after hydrolysis with hydrochloric acid and removal of protein, were carried out on those muscles used for total carbohydrate analysis. The mercuric chloride in the residue does not upset the results, which are of the same order when trichloroacetic acid is used for precipitating protein¹. In all the muscles where the change in glycogen during incubation was determined a small amount of glycogen was found after incubation. This was approximately the same as the residual glycogen found in the total carbohydrate determinations. It seems possible that this residual glycogen is of a different nature from the main bulk of that present in muscle, since it is not extracted during hydrolysis with hydrochloric acid and is not removed during incubation with phosphate buffer. This raises the question whether or not the correction for solubility in the alcohol used to precipitate the glycogen should be applied in calculating the amount of residual glycogen present, since, if the latter is of a different nature from the rest of the glycogen, it possibly has a different solubility in alcohol. In Table IX the results are shown both with and without the correction; in Table VIII the correction is used in the calculation of all the results.

Bicarbonate buffer. The results of estimations on muscles from autumn frogs of the same batch incubated in bicarbonate buffer are shown in Tables VIII (*d*) and IX (*b*). Although the decrease in total carbohydrate is in all cases except one greater than the increase in lactic acid, the discrepancy is not nearly so great as in the case of incubation in phosphate buffer, and moreover a large amount of carbohydrate is left in the muscle. An increase in hexosephosphate during incubation would tend to make the final figures for total carbohydrate low in comparison with the initial, and possibly this accounts for the greater decrease of total carbohydrate compared with increase of lactic acid. The increase in soluble carbohydrate in Table VIII (*d*) supports the idea of increase in hexosephosphate.

These results and the determination of glycogen and lactic acid agree with those of Meyerhof, of Laquer and of Embden, that lactic acid formation ceases whilst there is still carbohydrate left in the muscle. In two cases in Table VIII (*d*) the lactic acid increase exceeds the carbohydrate decrease calculated from the change in glycogen and alcohol-soluble carbohydrate, though in one it is less than the glycogen change taken alone. If, as is probable, the figures for alcohol-soluble carbohydrate are low, then the change in this fraction should be greater than appears in Table VIII (*d*). This change is always an increase and it is possible that were its full value known it would account for the difference between glycogen increase and lactic acid decrease.

¹ The reducing value after hydrolysis was estimated by the Hagedorn and Jensen method in all cases except two, in which the Shaffer-Hartmann method was used.

As is generally accepted, this difference between the action of the two buffer mixtures suggests that phosphate is required before all the carbohydrate can be converted into lactic acid. When the difference in the behaviour of total carbohydrate is considered it seems probable that in either case the chief source of lactic acid is glycogen, and that the large excess of carbohydrate found when the muscle is extracted with hydrochloric acid, as in this method of determining total carbohydrate, over that found using an alcoholic extraction, cannot form lactic acid at all. It may be noted in this connection that the trisaccharide of Lohmann does not give rise to lactic acid. From a comparison of the action of the two buffer mixtures it seems further very probable that this excess of carbohydrate does not react at all in a bicarbonate buffer, but is converted into a phosphate during incubation in a phosphate buffer. This phosphate may of course only be of a transitory nature.

Changes taking place in muscles poisoned with sodium moniodoacetate. Lundsgaard [1930] has shown that for muscles poisoned with sodium moniodoacetate although there is no increase in lactic acid content during anaerobiosis or when the muscle is fatigued, the glycogen content falls and the hexose-phosphate content rises proportionately. Lundsgaard has shown that the hexose-phosphate is in large part a diphosphate, and from the hydrolysis curve he concludes that it is probably identical with the hexosediphosphate of Harden and Young. This phosphate is much more easily hydrolysed than the monophosphate so that total carbohydrate should show little or no change during anaerobiosis or after fatigue. This was confirmed by experiment, the results of which are shown in Table X. The frogs were injected in the dorsal lymph sac with a 1 % solution of moniodoacetic acid neutralised to p_H 7 with sodium carbonate. 4 cc. per 100 g. body weight were given. After injection the frogs were placed in the refrigerator for 40 minutes so that the muscles should be in a resting condition when the frog was killed. The muscles were dissected on a board which was well cooled and surrounded by ice so that the changes taking place after death and not due to the treatment given should be a minimum. The control muscles were ground in a mortar with ice-cold alcohol or hydrochloric acid immediately after dissection and weighing. For these experiments it was considered better not to soak the muscles in oxygenated Ringer solution as the changes taking place are not yet fully worked out, and a continuous breakdown might be taking place in the Ringer solution instead of recovery. The muscles were fatigued by single induction shocks at the rate of about 2 per second in an atmosphere of hydrogen. The record of a lever attached to the muscles was taken and in all cases a curve typical of a poisoned muscle was obtained, that is the muscle became inexcitable after 100-200 shocks and remained in a contracted state. For anaerobiosis the muscle was hung in a tube containing hydrogen and either left at 16° for 24 hours, or placed in an incubator at 37° for 2 hours. Determinations were also made of the change in glycogen and alcohol-soluble carbohydrate and sugar in poisoned muscles. The glycogen change is of the same order as that found by Lundsgaard, but the increase in alcohol-soluble carbohydrate does not correspond

Table X.

Change in total carbohydrate, glycogen, alcohol-soluble carbohydrate and sugar, and lactic acid, in muscles poisoned with moniodoacetate, mg. per 100 g.

Frog	Muscle	Weight	Total carbohydrate initial final change	Glycogen initial final change	Alc.-sol. carbo- hydrate initial final change	Alcohol- soluble sugar initial final change	Lactic acid initial final change
(a) Fatigue in hydrogen.							
I	G	1.25	—	1200 964 - 236	17 31 + 14	6 20 + 14	20 22 + 2
II	G	1.10	1260 1237 - 23	—	—	—	51 50 - 1
III	G	0.77	1115 1115	—	—	—	29 48 + 19
IV	G	0.90	1082 1085 + 3	—	—	—	35 3 - 32
V	G	0.44	—	768 547 - 221	27 57 + 30	12 20 + 8	19 28 + 9
(b) Anaerobiosis in hydrogen.							
I	Gr	0.80	—	1325 795 - 530	42 92 + 50	46 48 + 2	16 30 + 14
I	Sm	0.30	—	738 653 - 85	43 103 + 60	—	43 28 - 15
II	S	0.20	720 709 - 11	—	—	—	11 30 + 19
II	Gr	1.10	1270 1040 - 230	—	—	—	51 50 - 1
III	Gr	0.85	—	704 281 - 423	12 73 + 61	11 84 + 73	8 7 - 1
V	Gr	0.30	878 905 + 20	—	—	—	20 62 + 42
(c) 2 hours in air.							
I	S	0.20	—	670 590 - 80	104 273 + 169	4 63 + 59	37 78 + 41

with the decrease in glycogen. The values obtained for the former may be low, owing to incomplete extraction of the carbohydrate present by alcohol. In this case the low values cannot be explained by supposing that there is hexosephosphate present which is incompletely hydrolysed, since there is no change in total carbohydrate content, where the time allowed for hydrolysis is less than that allowed in the case of the alcohol-soluble carbohydrate.

SUMMARY.

1. A modification of the method of estimating total carbohydrate of muscle by hydrolysing the whole muscle is described.
2. A modification of Bissinger's method of separating sugar from solution and estimating it quantitatively by precipitation with copper-lime and using the copper as the oxidising agent for sugar estimation is described.
3. The resting value of the total carbohydrate of isolated frog muscles estimated by this method is found to be much greater than the sum of the glycogen and alcohol-soluble carbohydrate.

4. During incubation in a phosphate buffer at p_H 8 this excess carbohydrate is removed from the muscle but does not appear as lactic acid. The lactic acid increase is in general somewhat greater than the decrease of the sum of glycogen and alcohol-soluble carbohydrate.

5. The excess carbohydrate is not removed during incubation in 2 % bi-carbonate buffer.

6. In muscles poisoned with monoiodoacetic acid, during anaerobiosis and fatigue the total carbohydrate content does not change significantly whilst the glycogen content falls. The lactic acid content rises very slightly on the average. The alcohol-soluble carbohydrate rises but not sufficiently to account for the fall in glycogen.

7. When the total carbohydrate of muscle is estimated by this method there is left in the muscle a small residual amount of glycogen, on the average 50 mg. per 100 g. muscle. This glycogen does not change significantly in amount during incubation in phosphate buffer. Approximately the same amount of glycogen is found remaining after incubation when the whole glycogen content of the muscle is estimated by Pfüger's method. It is suggested that this glycogen may be of a different nature from the main bulk of that present in muscle.

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REFERENCES.

- Anderson and Macleod (1930). *Biochem. J.* **24**, 1408.
 Barbour (1930). *J. Biol. Chem.* **85**, 29.
 Bissinger (1926). *Biochem. Z.* **168**, 421.
 — and Lesser (1926). *Biochem. Z.* **168**, 398.
 Boyland (1928). *Biochem. J.* **22**, 238.
 Dulière and Horton (1929). *J. Physiol.* **67**, 152.
 Eggleton and Eggleton (1929). *J. Physiol.* **68**, 193.
 Embden and Jost (1928). *Z. physiol. Chem.* **179**, 24.
 — and Zimmerman (1924). *Z. physiol. Chem.* **141**, 225.
 Folin (1926). *J. Biol. Chem.* **67**, 357.
 Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.
 Hanes (1929). *Biochem. J.* **23**, 99.
 Holmes (1929). *Biochem. J.* **23**, 1182.
 — and Gerard (1929). *Biochem. J.* **23**, 738.
 Kerly (1930). *Biochem. J.* **24**, 67.
 Laquer (1914). *Z. physiol. Chem.* **93**, 60.
 — (1921). *Z. physiol. Chem.* **116**, 169.
 Lesser (1920). *Biochem. Z.* **103**, 1.
 Loewi (1918). *Therap. Monatsh.* **32**, 350.
 Lohmann (1926). *Biochem. Z.* **178**, 444.
 Lundagaard (1930). *Biochem. Z.* **227**, 51.
 Meyerhof (1920). *Pfüger's Arch.* **185**, 11.
 — (1930). *Die chemische Vorgänge im Muskel* (Julius Springer, Berlin, 1930).
 — Lohmann and Meier (1925). *Biochem. Z.* **187**, 459.
 Ochoa (1930). *Biochem. Z.* **227**, 116.
 Parnas (1921). *Biochem. Z.* **116**, 71.
 — and Wagner (1914). *Biochem. Z.* **61**, 387.
 Simpson and Macleod (1927). *J. Physiol.* **64**, 255.
 Van Slyke (1917). *J. Biol. Chem.* **32**, 455.
 West, Scharles and Peterson (1929). *J. Biol. Chem.* **82**, 137.

LXXVII. EXPERIMENTS ON NUTRITION.

X. COMPARATIVE VITAMIN B₁ VALUES OF FOODSTUFFS. CEREALS II.

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IN our previous experiments [Plimmer, Rosedale, Raymond and Lowndes, 1927] on testing cereals for their vitamin B (B₁) value, rearing of young by pairs of adult pigeons was taken as the standard of comparison. Some data concerning the maintenance of adult birds were also then given. A greater amount of foodstuff containing vitamin B was needed for rearing than for maintenance. This fact has been again observed in subsequent experiments.

It was considered at that time that the standard of rearing was too high and not altogether satisfactory. Other factors might well be concerned in the hatching of eggs and rearing of young, such as the presence of fat-soluble vitamins which were not allowed for. The standard of maintenance for a period of at least 26 weeks was adopted in our trials with pulses and nuts [Plimmer *et al.*, 1929] with satisfactory results. In order to bring cereals into proper comparison it was necessary to test them again on this standard. The results of these experiments are herewith presented.

In addition we have made some experiments on the extraction of vitamin B from wheat germ and have again tested marmite for comparison with the cereals and for ascertaining if its value were the same after a period of 5 or 6 years.

The term vitamin B is now used to include several unknown factors. In these tests the symptoms of head retraction of pigeons have been taken as the sign of insufficient vitamin B, *i.e.* in the modern nomenclature vitamin B₁.

EXPERIMENTAL.

The experiments have been made with pairs of pigeons kept in cages on the roof of the building as in previous trials with pulses.

The food was made into pills of white flour and the test grain which was ground finely in a coffee mill. In the earlier experiments cod-liver oil was not included, but later 1 % was added so that the birds should not suffer from a shortage of fat-soluble vitamins. Fishmeal was included to supply protein and mineral salts.

The results are recorded in a series of tables in the same form as in our earlier experiments with pulses.

Wheat.

In our previous paper it was reported that a pair of adult pigeons had been maintained on a diet containing 50 % whole wheat flour for 45 weeks, and that another pair (Nos. 93 and 26/27) had been kept on 40 % whole wheat flour for 23 weeks. This pair has been continued on the same diet for 78 weeks. They lost a little weight in the first 20 weeks, but at 40 weeks were above their original weight. Then followed a small decline and after 78 weeks they were slightly below their original weight. The hen laid some eggs at the start, but not again till the 46th week. On reducing the quantity of whole wheat to 30 % both birds lost weight, became ill, showed polyneuritis and died. 40 % wheat was clearly the minimum for maintenance (Table I).

Table I. *Whole wheat.*

Date	Time in weeks	Diet			Weights, g.		Remarks
		Whole wheat	White flour	Fish- meal	Cock No. 93	Hen No. 26/27	
30. iii. 27					445	365	
to 17. viii. 27	20	40	55	5	385	295	Six eggs. One squab hatched and reared, but poor growth. Other eggs deserted
to 4. i. 28	40				530	430	
to 23. v. 28	60				440	355	
to 26. ix. 28	78				400	330	Twelve eggs; all deserted
to 24. x. 28	4	30	65	5	335	305	Both showed retracted head positions and died

Bran.

Two pigeons had previously been maintained on 30 % bran for 32 weeks. The experiment has been repeated and young have been reared on this quantity. The same pigeons were then maintained on 20 % bran and a pair of young was reared. On reducing the quantity to 10 % both birds lost weight and showed polyneuritis. 10 % bran was insufficient as verified by testing with another pair of birds (Table II).

Middlings.

Previously, a pair of pigeons had been maintained on a diet containing 30 % of middlings mixed with white rice for 16 weeks. In these experiments some of the middlings on account of the small particles could not be picked up so that white flour pills were substituted; a longer time for maintenance was also essential. The trial was started with a pair of birds on 30 % middlings which was reduced to 20 % after a short period on whole wheat. Cod-liver oil (1 %) was now mixed with the food to ensure the presence of fat-soluble vitamins. They were maintained on this diet for 27 weeks and again after an interval on whole wheat the amount of middlings was reduced

Table II. *Bran.*

Date	Time in weeks	Diet			Cod- liver oil	Weights, g.		Remarks
		Bran	White flour	Fish- meal		Cock No. 75	Hen No. 76	
8. v. 29 to 13. xi. 29	27	30	65	5	0	510	360	Eight eggs laid, four hatched and young reared. Four hatched and young died
11. xii. 29 to	4	On whole wheat				595	410	Ten eggs laid; first two hatched and young reared, Nos. 4 and 6 below. Eight deserted
18. vi. 30 to 2. vii. 30	27	20	74	5	1	535	405	
23. vii. 30	2	10	84	5	1	455	355	Cock showed head retraction and died
	5					—	305	Hen showed head retraction and died
29. i. 30 to 6. viii. 30	28					No. 4 95	No. 6 105	Squabs reared on 20 % bran
28. v. 30 to 9. vii. 30	6	20	74	5	1	460	425	
23. vii. 30	8	10	84	5	1	No. 9 425	No. 29 430	
						245	310	Cock showed paralysis and died
						—	245	Hen showed head retraction and died

to 10 %. They were quite well on this quantity, but on reducing the amount to 5 % they lost weight and showed the symptoms of polyneuritis.

The experiment was confirmed with another pair of pigeons, starting at 10 % middlings. The insufficiency of 5 % middlings was verified with a third pair of pigeons. The details are shown in Table III. The sample of middlings would thus appear to contain a considerable proportion of wheat germ, the value of which we had found to be 6 % for maintenance. Other samples of middlings may not have such a high vitamin B value. We have not tested any other samples.

Wheat germ.

For comparison with whole wheat, middlings and bran some further trials have been made with wheat germ. No difference was previously noticed between fresh and heated wheat germ. In these experiments "bemax" was used as source of wheat germ. Maintenance was secured on 6 %, but on prolonging the test both birds died without showing the typical signs of polyneuritis. Two pairs of birds were maintained on 7 % for 26 weeks and one pair was kept for 53 weeks. The details are shown in Table IV. The material had thus the same value as the wheat germ of the previous trial.

Extraction of wheat germ with solvents. With a view of extracting and concentrating vitamin B₁ some experiments were first made to ascertain the completeness of the extraction with solvents. Fat solvents, such as ether, chloroform, light petroleum do not extract vitamin B₁. The residue after

Table III. *Middlings.*

Date	Time in weeks	Diet				Weights, g.		Remarks
		Midd- lings	White flour	Fish- meal	Cod- liver oil	Cock No. 15	Hen No. 44	
8. v. 29 to		30	65	5	0	370	375	Seven eggs. First two hatched and young reared; one died later
13. xi. 29 to	27	On whole wheat				455	445	Other eggs deserted
11. xii. 29 to	4					410	435	No eggs
12. iii. 30 to	13	20	75	5	0	415	450	No eggs
18. vi. 30 to	14					430	425	Nine eggs. One hatched, squab died. Others de- serted
17. xii. 30 to	26	10	84	5	1	455	420	Six eggs at beginning, none later
14. i. 31	4	5	89	5	1	335	335	Hen showed head retraction and was cured. Cock had paralysis and died
28. v. 30 to						No. 85 460	No. 86 420	
31. xii. 30 to	31	10	84	5	1	550	435	Six eggs; all deserted
28. i. 31	4	5	89	5	1	390	320	Cock showed head retrac- tion and was cured. Hen had head retraction and died
5. xi. 30 to						No. 100 420	No. 1557 375	
10. xii. 30	5	5	89	5	1	300	315	Both showed head retrac- tion and were cured

Table IV. *Wheat germ.*

Date	Time in weeks	Diet			Weights, g.		Remarks
		"Bemax"	White flour	Fish- meal	Cock No. 329	Hen No. 327	
28. ix. 27 to					330	330	
18. iv. 28 to	29	6	89	5	355	340	Six eggs; deserted Cock died
27. vi. 28	39						Hen died. Both birds at <i>p.m.</i> examination had very wet tissues and very large hearts
1. viii. 28 to					No. 88 380	No. 80 410	
20. ii. 29	29	7	88	5	465	495	No eggs during winter months
26. ix. 28 to					No. 89 390	No. 49 345	
20. iii. 29 to	25				450	385	Four eggs; deserted
16. x. 29	30				410	365	

extraction, as shown in Table IV *a*, fed to pigeons in an amount of 6 % gave maintenance for 26 weeks. Extraction with absolute alcohol subsequent to

Table IV *a*.

Date	Time in weeks	Diet			Weights, g.		Remarks
		Ether- extracted "bemax"	White flour	Fish- meal	Cock No. 54	Hen No. 75	
28. ix. 27					385	400	
to		6	89	5			
24. iv. 28	30				365	350	
to							
26. ix. 28	52				350	320	Two eggs in 43rd week; deserted
		Ether + abs. alc.- extracted "bemax"			No. 64	No. 520	
8. ii. 28					465	495	
to		10	85	5			
8. viii. 28	26				440	470	Eighteen eggs; deserted
to			On wheat				
29. viii. 28	3				420	455	Two eggs; deserted
to		6	89	5			
17. x. 28	7				350	390	Two eggs; deserted. Cock had head retraction. Cured
to							
31. x. 28	9				—	305	Hen died. Had large heart
		Light petroleum + abs. alc.- extracted "bemax"			No. 88	No. 80	
20. ii. 29					465	495	
to		7	88	5			
21. viii. 29	26				435	455	Twelve eggs; deserted
		Abs. alc.- extracted "bemax"			No. 26/28	No. 58	
13. ii. 29					445	360	
to		50	47.5	2.5			
7. viii. 29	25				395	330	No eggs
		Petroleum + 50 % alc.- extracted "bemax"			No. 93	No. 50	
20. iii. 29					420	470	
to		7	88	5			
24. iv. 29	5				275	305	Both had head retraction
					No. 26/28	No. 58	
4. ix. 29					430	335	
to		21	74	5			
9. x. 29	5				315	275	Both had head retraction
					No. 89	No. 49	
20. xi. 29					450	385	
to		42	53	5			
11. xii. 29	3				350	300	Both had head retraction

these solvents removed only a small quantity of vitamin B₁. Pigeons were maintained with 10 % of the residue in the diet, but failed on 6 %. Complete extraction was effected with 50 % alcohol. The residue was tested in amounts of 7, 21 and 42 % of the diet. The birds showed symptoms of polyneuritis at each level in 3 to 5 weeks. The vitamin was present in the 50 % alcoholic

extract. It is thus not necessary to use acid alcohol for the extraction. Further experiments with the alcoholic extract have been discontinued as similar work is being carried out by Guha and Drummond [1929].

Barley.

55 % of whole barley in the diet with white rice was the smallest amount used previously and the pigeons were maintained for 42 weeks. Two pigeons were started on 40 % barley with white flour to compare with wheat. After 7 weeks they were found to be two cocks and a hen was substituted for one of them. They were maintained for 35 and 27 weeks respectively. After an interval on whole wheat, the barley was reduced to 30 %. Both suffered from polyneuritis. The vitamin B₁ value of barley was thus the same as that of wheat.

Table V. *Barley.*

Date	Time in weeks	Diet				Weights, g.		Remarks
		Barley	White flour	Fish- meal	Cod- liver oil	Cock No. 333	Hen No. 54	
5. ix. 28						405	—	
to		40	55	5	0	420	345	
7. xi. 28	11							
to						415	355	Four eggs; one hatched,
15. v. 29	29							squab died
to			On whole wheat					Two eggs; one hatched
3. vii. 29	7							and reared
to		30	65	5	0			Four eggs; deserted
14. viii. 29	6					320	325	Cock had head retraction
to								and died
11. ix. 29	10					—	210	Hen had head retraction
								and died

Malt extract.

Cooper [1914] examined several malt extracts for vitamin B by the curative method and found that two samples had curative action. Stammers [1924] and Southgate [1924] have also found vitamin B in malt. Randoin and Lecoq [1927] on substituting malt extract for dextrin to the extent of 66 % of the diet obtained cure and prevention of polyneuritis. Bacharach and Allechorne [1928] considered malt extract a rich source of vitamin B and of almost equal value to the cereal from which it was made.

Malt extract had not been previously tested by us. With the pill method of feeding pigeons it was easy to incorporate the sticky material with white flour and try its value in comparison with cereals. The extract tested was the same as is used for patients in St Thomas's Hospital. Two trials were made with 40 % malt in the diet, three with 30 % and one with 20 %. The birds suffered from polyneuritis on 20 %, but were maintained on 30 % and 40 % for 23 and 27 weeks. The value of the malt extract was thus slightly greater than that of barley. The data are given in Table VI.

Table VI. *Malt extract.*

Date	Time in weeks	Diet				Weights, g.		Remarks
		Malt extract	White flour	Fish- meal	Cod- liver oil	Cock No. 24	Hen No. 70	
29. v. 29						375	375	
to 11. ix. 29	15	40	55	5	0	260	295	Birds ate very little and lost weight. Both not well. Cock had head retraction and was cured. Both got better. No eggs
19. ii. 30	23					415	410	
to 13. viii. 30	25	30	65	5	0	345	305	Hen paralysed and died
to 27. viii. 30	27					310	—	Cock had head retraction and died
						No. 343	No. 60	
23. x. 29						465	410	
to 19. iii. 30	21	40	55	5	0	440	375	Ten eggs; all deserted
to 7. v. 30	7	40	54	5	1	425	365	
to 23. vii. 30	11	30	64	5	1	410	320	Four eggs; deserted
to 17. ix. 30	19					390	305	Cock had head retraction and died. Hen was paralysed and died
						No. 98	No. 62	
6. xi. 29						490	405	
to 8. i. 30	9	30	64	5	1	415	375	Cock had head retraction and died
to 26. iii. 30	20					—	330	Hen had head retraction and died
						No. 42	No. 38	
19. iii. 30						455	300	
to 21. v. 30	9	20	74	5	1	340	245	Both showed head retraction and were cured

Oatmeal and oats.

Previous work led to the conclusion that rearing was not possible on oatmeal without an addition of marmite, though adult pigeons could be maintained for 40 and 52 weeks on 95 % oatmeal. Maintenance experiments with oatmeal were therefore essential for proper comparison with other cereals. Maintenance was found on a diet containing 50 % oatmeal for 31 weeks, and also with 40 % oatmeal. This result was confirmed with a second pair of birds. On reducing the amount of oatmeal to 30 %, the cock bird showed polyneuritis in 28 weeks. 30 % oatmeal was tested again with another pair of pigeons. In this case the hen showed polyneuritis, but not the cock. There was failure on 20 % oatmeal. Maintenance was thus possible on 30 % oatmeal as the minimum quantity, but it is not certain and we ought to take 35 % as the proper minimum quantity. Oats had the same value as oatmeal. The data are given in Table VII.

Table VII. *Oatmeal.*

Date	Time in weeks	Diet				Weights, g.		Remarks
		Oat- meal	White flour	Fish- meal	Cod- liver oil	Cock No. 56	Hen No. 14	
29. ii. 28 to		50	45	5	0	415	375	
3. x. 28	31					400	390	Twelve eggs. Ten deserted. Hatches from last two, but squabs died
1. viii. 28 to		40	55	5	0	No. 57 465	No. 6 385	
27. ii. 29	29					540	515	Two eggs at beginning, hatched but squabs died No more eggs
1. v. 29 to		40	55	5	0	No. 236 475	No. 99 390	
13. ii. 29 to	28					525	445	Ten eggs. Four hatched and reared with poor growth and weak. Two hatched and died. Four deserted
8. i. 30 to		30	On whole wheat 64	5	1	555	405	
23. vii. 30 to	28					425	445	Ten eggs. Two hatched; squabs died. Others de- serted
6. viii. 30 to	30	(Oats) 30	64	5	1	475	415	Cock showed head retrac- tion and was cured
1. x. 30 to	8					380	400	Four eggs; deserted. Cock showed head retrac- tion and was cured. Hen kept on diet
11. ii. 31	27					—	410	
16. vii. 30 to						No. 1156 410	No. 35 400	
29. x. 30 to	15	30	64	5	1	410	330	Two eggs; deserted Hen paralysed and died.
11. ii. 31	15					420	—	Cock continued on diet
30. vii. 30 to						No. 56 395	No. 38 305	
17. ix. 30	7	20	74	5	1	285	215	Both showed head retrac- tion. Hen died; cock cured

Buckwheat.

Buckwheat had not been found a satisfactory grain for rearing, but a pair of pigeons had been maintained on 70 % buckwheat in the diet for 31 weeks. Proper comparison with other cereals was needed. A pair of pigeons was kept for 29 weeks first on 40 %, then on 30 % and later on 20 %. On reducing the amount of buckwheat to 10 %, both birds showed polyneuritis and died. The data are in Table VIII. Buckwheat is thus slightly richer in vitamin B than the other grains.

Table VIII. *Buckwheat.*

Date	Time in weeks	Diet				Weights, g.		Remarks
		Buck- wheat	White flour	Fish- meal	Cod- liver oil	Cock No. 334	Hen No. 335	
10. x. 28 to 1. v. 29	29	40	55	5	--	375	345	Five eggs; deserted
29. v. 29 to 18. xii. 29	4	On whole wheat				360	340	Two eggs; deserted
29. v. 29 to 6. viii. 30	29	30	65	5	—	350	335	Seven eggs; deserted
18. xii. 29 to 12. xi. 30	29	20	74	5	1	410	400	Eight eggs; deserted
6. viii. 30 to 17. ix. 30	29	On whole wheat				365	340	One egg; deserted
17. ix. 30 to 12. xi. 30	6	10	84	5	1	345	320	Two eggs; deserted
12. xi. 30 to 26. xi. 30	8					255	280	Cock had head retraction and died
26. xi. 30	10					—	255	Hen showed paralysis and died

Maize.

The experiment with maize carried out previously was maintenance on 60 % maize with 35 % white flour for 18 weeks. The same pair of pigeons after an interval on whole wheat was tested with 40 % maize. As seen from Table IX they showed polyneuritis in about 20 weeks. This amount was thus insufficient. No further experiment was made, as previously a pair had been maintained on 50 % maize for 23 weeks.

Table IX. *Maize.*

Date	Time in weeks	Diet			Weights, g.		Remarks
		Maize	White flour	Fish- meal	Cock No. 26/4	Hen No. 26/5	
5. x. 27 to 15. ii. 28	19	40	55	5	445	395	Ten eggs; deserted
15. ii. 28 to 22. ii. 28	20				380	290	Hen had head retraction and died
22. ii. 28					360	—	Cock had head retraction and died

Rye.

With rye it had been observed that a pair of birds could be maintained for 31 weeks on a diet containing 45 %. The next trial was to use 40 %. The first cock bird was not satisfactory, but a second was kept on this amount for 26 weeks and the hen for a total of 47 weeks. On reducing the amount of rye to 30 %, both birds showed polyneuritis. This was confirmed with another pair and another hen put in with the cock after the first hen had died. The cock bird seemed rather resistant to shortage of vitamin B and only showed symptoms after 39 weeks. During the last period of 6 weeks his food consumption was measured and was from 10 to 13 g. daily as compared with a normal consumption by healthy birds of 30 to 40 g. daily. On a small consumption of food it has often been noticed that pigeons will live for long

periods before they show head retraction, or die showing only symptoms of paralysis. The data are in Table X.

Table X. *Rye*.

Date	Time in weeks	Diet			Weights, g.		Remarks
		Rye	White flour	Fish- meal	Cock No. 77	Hen No. 25/27	
27. vi. 28 to 12. ix. 28	11	40	55	5	405	360	
					330	365	Cock changed
					No. 87 395	—	
14. xi. 28	10				475	385	Four eggs; deserted Cock escaped
					No. 64 420	—	
22. v. 29	26 (47)				425	345	Fourteen eggs; deserted
19. vi. 29		On whole wheat			420	380	
14. viii. 29	8	30	65	5	340	275	Two eggs; deserted Cock had head retraction and died
28. viii. 29	10				—	255	Hen had head retraction and died
					No. 167 415	No. 82 320	
5. ix. 28 to 28. xi. 28	12	30	65	5	340	250	Hen had head retraction and died
						No. 94 385	
9. i. 29	18				440	385	
1. v. 29	13				320	285	Hen had head retraction and was cured
5. vi. 29	39				285	—	Cock had head retraction and was cured

Dari and millet.

Rearing experiments only were made previously with these grains. The smallest amounts with white rice were 55 %. Tests have now been made using white flour pills with 40 % of the grains. On this amount the pigeons

Table XI. *Dari*.

Date	Time in weeks	Diet				Weights, g.		Remarks
		Dari	White flour	Fish- meal	Cod- liver oil	Cock No. 84	Hen No. 73	
7. xi. 28 to		40	55	5	0	535	355	
15. v. 29 to	27					515	385	Four eggs; one hatched, squab died
19. vi. 29	5	On whole wheat				560	370	Four eggs; one hatched and squab reared
20. xi. 29	22	30	65	5	0	435	320	Both birds had head retrac- tion and were cured

were maintained with both grains. They failed on 30 % dari, and on 20 % millet. The data are in Tables XI and XII.

Table XII. *Millet.*

Date	Time in weeks	Diet				Weights, g.		Remarks
		Millet	White flour	Fish- meal	Cod- liver oil	Cock No. 100	Hen No. 1557	
6. iii. 29 to 9. x. 29	31	40	55	5	0	395	360	Twelve eggs; three hatched, one squab reared, two died
6. xi. 29 to 4. vi. 30	4		On whole wheat			410	360	Two eggs; deserted
23. vii. 30 to 3. ix. 30	30	30	64	5	1	445	380	Eight eggs; one hatched, squab died, others deserted
10. ix. 30	7		On whole wheat			415	375	Two eggs; hatched and squabs reared
	7	20	74	5	1	410	350	
	6					270	305	Cock had head retraction and was cured
	7					280	270	Hen had head retraction and was cured

Rice.

It was of interest and importance to compare the value of brown rice, *i.e.* rice with its pericarp, with that of other cereals. Tests were started with 40 % rice. On this amount the pigeons were maintained. but they suffered from polyneuritis on 30 %. Such rice has thus the same value in vitamin B as wheat and other cereals. The results are in Table XIII.

Table XIII. *Rice.*

Date	Time in weeks	Diet				Weights, g.		Remarks
		Brown rice	White flour	Fish- meal	Cod- liver oil	Cock No. 88	Hen No. 80	
25. ix. 29 to 16. iv. 30	29	40	55	5	0	445	455	Six eggs; deserted
28. v. 30 to 4. vi. 30	6	30	64	5	1	430	475	
25. vi. 30 to 3. ix. 30	10					370	420	Hen died, new hen used
	13						No. 95	
						395	365	
						275	350	Cock had head retraction and died
						—	285	Hen had head retraction and died

Marmite.

Partly from our experiments and partly from experiments of other workers the belief has arisen that marmite has not such a high content of vitamin B as it had 8 or 9 years ago. Our first trials [Plimmer, Rosedale, and Raymond, 1923] indicated that 3 % marmite was sufficient to keep a pair of pigeons in health. These trials were of short duration, from 5 to 6 weeks, and are not strictly comparable with those of our present standard of maintenance for at

least 26 weeks. Also, white rice was used instead of white flour, which allowed the birds to reject any grains not properly coated with marmite. It is therefore possible that this amount would not have sufficed in a long trial. Later [Plimmer, Rosedale and Raymond, 1927, pp. 929-931] it was found that amounts varying from 4 to 8 % of different yeast extracts, again tested for short periods, sufficed. The first trial with marmite with long maintenance as the standard indicated that 8 % was the minimum amount.

Since marmite is so often used as the source of vitamin B in experimental diets, it was considered of distinct importance to make tests with marmite of recent manufacture. It was not possible to test marmite manufactured 10 years ago so as to obtain an exact comparison. Two samples have been kindly supplied by the makers, prepared from the same yeast. The first

Table XIV. *Marmite*.

Date	Time in weeks	Diet				Weights, g.		Remarks
		Marmite. Ct. marmite	White flour	Fish- meal	Cod- liver oil	Cock No. 89	Hen No. 49	
15. i. 30 to 26. iii. 30	10	8	87	5	0	455	380	
						455	305	Hen died from burst egg in oviduct
							No. 43	
2. iv. 30 to 16. vii. 30	11 26, 15	8	86	5	1	460	445	Eight eggs; one hatched, squab died. Others deserted
		6	88	5	1	435	460	Two eggs, one hatched, squab died
11. ii. 31 to 25. ii. 31	30 2	4	90	5	1	430	445	
						305	375	Both birds had head retraction. Cock cured, hen died
						No. 96	No. 2	
1. x. 30 to 19. xi. 30	8	4	90	5	1	370	295	
						340	315	Cock had head retraction and died
28. i. 31	17					—	—	Hen paralysed and died
		K. marmite				No. 18	No. 39	
15. i. 30 to 26. iii. 30	10	8	87	5	0	530	505	Four eggs; deserted
		8	86	5	1	515	510	Eight eggs; deserted
16. vii. 30 to 11. ii. 31	16 30	6	88	5	1	475	455	Six eggs; deserted
		4	90	5	1	520	500	
4. iii. 31 to 18. iii. 31	3 5					470	400	Hen had head retraction and cured
						350	—	Cock had head retraction and cured
						No. 23	No. 3	
1. x. 30 to 19. xi. 30	8	4	90	5	1	355	325	
						285	275	Cock had head retraction and cured
28. i. 31	17					—	235	Hen paralysed and died

(Ct. marmite) was similar to the material of the last few years, the second (K. marmite) was material made from a clarified extract. This is the present method of manufacture. As may be seen from Table XIV both the samples had the same value. 6 % in the diet was the minimum for maintenance. This compares with 8 % found in 1925. There is thus no essential change in the vitamin B value of marmite in a period of 6 years. It may be noted that marmite if kept for such long periods may lose value, as we observed in 1923. Freshly manufactured marmite seems of approximately the same value from year to year.

Baker's yeast.

The amount of baker's yeast required to supply vitamin B was found previously to be above 10 % of the diet. An experiment with 12 % in the diet sufficed for maintenance of two pigeons for 31 weeks. This value corresponds with its moisture content and reckoned on dry weight its value is the same as that of dried brewer's yeast, 4 % of the diet.

DISCUSSION.

These experiments do not differentiate between the several factors of the vitamin B complex. They give essentially the vitamin B₁ or antineuritic value. The vitamin B₂ value of cereals has been examined by Aykroyd and Roscoe [1929] and has been found to be very small. It was presumably supplied in the fishmeal of our diets and is probably not needed by pigeons according to the work of Carter, Kinnersley and Peters [1930]. If vitamin B₃ is to be regarded as necessary for the maintenance of weight, its amount in cereals and pulses must be in close correspondence with the amount of vitamin B₁. Reference to the weights of the pigeons in our tables shows that weight is maintained over the long periods of 26 or more weeks and loss of weight occurs as soon as the amount of cereal is reduced below the maintenance quantity, followed in a few weeks by the symptoms of head retraction in most cases and paralysis in the others. There was also invariably a fall in food consumption which really accounts for the loss in weight. Occasionally a bird has lived for many weeks at a much lower weight level and with a very small food consumption. The general metabolism of these birds is less and a smaller amount of vitamin B₁ or B₃ or both is then required. The vitamin B complex is wanted for the health of the bird to replenish the store in every cell of the body. At the low weight level the bird eventually dies unless cured, but, if cured, there is a large food consumption and gain in weight. *Post mortem* examination of our dead birds has almost invariably shown that the hearts are very large and flabby, and frequently the tissues of the birds are wet. Maintenance of weight may be due to such a wet condition of the tissues, and should be distinguished from increase of tissue substance.

SUMMARY.

Adopting the same standard of comparison as in our work on pulses and nuts, the comparative vitamin B values of cereals are as follows:

	Percentage amount in diet for maintenance	Comparative vitamin B value
Dried yeast	4	100
Marmite	6	67
Wheat germ ("bemax")	6-7	62
Middlings	10	40
Baker's yeast	12	33
Bran	20	20
Buckwheat	20	20
Millet	30	13
Oatmeal	35	11
Wheat	40	10
Barley	40	10
Malt	40	10
Rye	40	10
Dari	40	10
Brown rice	40	10

Cereals are thus not quite so rich in vitamin B as pulses, which had a comparative figure of 13.

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REFERENCES.

- Aykroyd and Roscoe (1929). *Biochem. J.* **23**, 483.
 Bacharach and Allchorne (1928). *Biochem. J.* **22**, 313.
 Carter, Kinnersley and Peters (1930). *Biochem. J.* **24**, 1844.
 Cooper (1914). *J. Hyg.* **14**, 12.
 Guha and Drummond (1929). *Biochem. J.* **23**, 880.
 Plimmer, Rosedale and Raymond (1923). *Biochem. J.* **17**, 772.
 ——— (1927). *Biochem. J.* **21**, 913.
 ——— and Lowndes (1927). *Biochem. J.* **21**, 1141.
 ——— Raymond and Lowndes (1929). *Biochem. J.* **23**, 546.
 Randoin and Lecoq (1927). *Bull. Soc. Chim. Biol.* **9**, 49.
 Southgate (1924). *Biochem. J.* **18**, 769.
 Stammers (1924). *Biochem. J.* **18**, 13.

LXXVIII. STRUCTURE AND ENZYME REACTIONS.

IX. THE SYSTEMS AMYLASE-STARCH-GELATIN AND UREASE-UREA-GELATIN.

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THE previous parts of this series were devoted to the study of the influence of adsorption on the velocity of enzymic reactions in heterogeneous systems. In the present paper experiments on the influence of the state of the sorbent, of its viscosity, occlusion and aggregation (flocculation) will be described.

Increase in the viscosity of the medium in which an enzymic reaction is proceeding in a heterogeneous system should lead to retardation of the velocity of reaction in two ways, by reduction of the number of collisions taking place in unit time between the free enzyme and substrate, and between the enzyme and substrate adsorbed on different colloidal particles. The effect of increasing viscosity was studied in the systems amylase-starch-gelatin and urease-urea-gelatin, differing in that the first system contains a colloidal, slowly diffusing substrate, whilst the substrate of the second system is a rapidly diffusing crystalloid. The phenomenon of occlusion in relation to the velocity of reaction was next studied in the above systems. As the state of aggregation of gelatin increases, monomers, together with adsorbed enzyme and substrate particles, cohere, yielding polyons, which may then further aggregate, finally to yield either a coagulum or a jelly. As a result of this process the enzyme or substrate or both may be enclosed within the aggregates, and, should the structure of these be such as to exclude entry of larger particles, may be excluded from reaction. The system amylase-glycogen-caseinogen, described in the preceding part of this series [Przyłęcki and Gurfinkel, 1930] affords an example of occlusion with consequent inhibition of reaction.

EXPERIMENTAL.

I. THE SYSTEM AMYLASE-STARCH-GELATIN.

Starch solution was prepared from starch paste (Merck's soluble starch), which was poured into boiling water and boiled for 30 min. under a reflux condenser. Gelatin solution was prepared by dissolving gelatin (Coignet Père et Cie, Paris) at 40°; the p_H of the solution was 5.89. Merck's amylase ("Diastase absolut") dissolved in saline solution and filtered was used in.

these experiments. The p_H of all systems was fixed by addition of Sørensen's phosphate buffer solutions (p_H 5.89). Reducing sugar was determined by Bertrand's method and starch by Pflüger's method for glycogen.

(i) *Determination of sugar in the presence of gelatin.*

In view of the observation that the results obtained by Bertrand's method for reducing sugars are greater in aqueous than in gelatin solution, it was first necessary to determine whether the difference depended on the concentrations of sugar and of gelatin, on the time during which these were in contact, or on the temperature at which the systems were maintained.

0.3 % solutions of glucose or maltose, containing 1, 2, 3 or 4 % of gelatin, were left for 2 hours at 30°, after which the sugar content was compared with that of similar systems which had been allowed to stand for 10 min. at 15°; the results indicate that neither the duration of contact nor the temperature affects the results obtained.

The effect of varying the concentrations of sugar and of gelatin was studied in the following way (Table I). The sugar contents of 20 cc. of 0.1, 0.2, 0.3 and 0.4 % glucose or maltose in 5 % gelatin solution, and of 0.2, 0.4, 0.6 and 0.8 % maltose in 0.5 % gelatin solution were determined; in all cases the values found were lower than in aqueous solution, and the difference varied irregularly with the concentration of sugar, from 51.5 % for 0.1 % maltose

Table I. *Influence of concentration of sugar on the values found using Bertrand's method in the presence of gelatin.*

20 cc. were taken for the determination.

		% concentration of sugar												Conc. of gelatin %
		0.1		0.2		0.3		0.4		0.6		0.8		
Sugar	mg. found	% diff.	mg. found	% diff.	mg. found	% diff.	mg. found	% diff.	mg. found	% diff.	mg. found	% diff.		
Maltose	9.7	51.5	30.5	23.7	50	16.6	64	20	—	—	—	—	5	
"	—	—	39	2.5	—	—	75	6.25	83	30	142	11.2	0.5	
Glucose	12.5	37.5	26.7	33.3	48	20	52	35	—	—	—	—	5	

in 5 % gelatin to 20 % for 0.4 % maltose. In systems in which the concentration of sugars was maintained constant (0.3 %), but that of gelatin was varied (1.25–7.5 %), it was found that the proportion of sugars found by Bertrand's method diminished with the concentration of gelatin, the difference increasing in the case of maltose from 1.8 % in 1.25 % to 30.4 % in 7.5 % gelatin; the corresponding figures for glucose were 7.5 % and 28.3 % (Table II).

The following experiments were next performed in order to determine whether the above effect was due to combination of gelatin with sugar, with the production of a compound of lower reducing capacity, or to combination with cuprous oxide, with consequent prevention of precipitation. 15 cc. of 0.3 % maltose solution were added to 40 cc. of Bertrand's solution, and the mixture was heated to boiling. As soon as the precipitate of cuprous oxide

Table II. *Influence of concentration of gelatin on the reducing sugar content as determined by Bertrand's method.*

The sugar content was 0.3 % and 20 cc. were taken for the determination.

Conc. gelatin	mg. glucose found	% difference	mg. maltose found	% difference
0	60.0	—	57.5	—
1.25	55.5	7.5	56.5	1.8
2.5	53	11.7	55	4.3
5	48	20	50	11.1
7.5	33	28.3	40.5	30.4

had commenced to form, 5 cc. of 8 % gelatin were added to one series of flasks, and 5 cc. of water to another, after which the solutions were boiled for a further 3 min., and cuprous oxide determined as usual. Practically identical results were obtained in all cases (42 mg. where gelatin was added, and 41.8 mg. in its absence). It follows that cuprous oxide does not combine with gelatin, nor is its precipitation prevented by gelatin, under the conditions of the above experiment.

Solutions containing maltose and gelatin were next subjected to ultra-filtration at 30° through parchment membranes, using a Zsigmondy apparatus. The membranes used were not completely impermeable to gelatin; for the purpose of this experiment it was considered sufficient if the filtrate gave no precipitate on addition of alcohol, as harder filters were partially impermeable to maltose, with consequent dilution of sugar in the filtrate. The results obtained, using 0.3 % maltose in 2 % gelatin solution and taking 10 cc. of filtrate or solution for each determination, indicate that compound-formation does not take place, 34.8 mg. of maltose being found in 10 cc. of the filtrate of the gelatin solution, and 33.3 mg. in the filtrate from solutions not containing gelatin, whilst the corresponding values for unfiltered solutions are 32.0 and 38.7. The cause of the lower values obtained by Bertrand's method for reducing sugar in the presence of gelatin is not therefore apparent.

(ii) *Inhibition of action of amylase on starch.*

(a) *Kinetics of starch-gelatin-amylase systems.* It was at first attempted to determine the velocity of decomposition of starch by amylase, in the presence of various concentrations of gelatin, by comparing the reducing sugar content of such systems with that of systems not containing gelatin. The results obtained were, however, not reproducible, in spite of the application of every precaution, and it was found necessary in certain cases to determine not reducing sugar produced but residual starch, using Pflüger's method for glycogen.

Series of systems, containing 10 cc. of 0.5 or 1 % starch solution, 5 cc. of gelatin solution, and 1 cc. of 0.1 % amylase or water were prepared, and the starch content was determined after keeping for 2 hours at 30°. The amount of starch decomposed was 37–38 mg. in systems containing 0.4 % of

gelatin, 30.3 mg. in 5 %, 29.3 mg. in 6 %, and 24 mg. in 9 % gelatin, representing a percentage retardation of respectively 20, 22 and 36 (Table III).

Table III. *Concentration of gelatin and velocity of enzymic hydrolysis of starch.*

Conc. starch %	Conc. gelatin %	mg. starch decomposed	
		In absence of gelatin	In presence of gelatin
0.63	1	83	83
		89	88
"	2	83	81
		89	90
"	3	83	85
		89	89
"	4	83	79
		89	89
"	5	83	81
		89	87
"	6	76	70
		84	79
"	7	76	72
		84	78
"	8	76	74
		84	78
"	9	76	76
		84	79
"	10	76	71
		84	76
0.32	1	38	38
"	2	"	38.6
"	3	"	38
"	4	"	37
"	5	"	30
"	7	"	29
"	9	"	24

(b) *Diffusion of starch and of amylase through gelatin gel.* Starch-gelatin gels were prepared by cooling mixtures of 15 cc. of 8 % gelatin and 10 cc. of 1 % starch to 0°, 15 cc. of water were then poured on the gels, which were left for 2, 4, 6 and 12 hrs. at 0°, after which the aqueous layer was examined for starch. This was in all cases found to be absent, indicating that starch does not diffuse through gelatin gels under the above experimental conditions. Analogous experiments in which 0.2 % amylase was taken in place of starch solution and in which the amylolytic power of the aqueous layer was determined showed that similarly amylase does not diffuse through gelatin gel.

(c) *Velocity of reaction in the presence of starch or amylase contained in gelatin gel.* Flasks were prepared containing 15 cc. of 3 % gelatin and 10 cc. of water or 1 % starch solution, and the solutions were left for 1, 2, 8 and 24 hrs. at 16°. 10 cc. of 1 % starch solution were then added to those flasks to which it had not previously been added; and 10 cc. of water to the remainder, and all flasks were allowed to stand for 1 hr. at 16°, after which 10 cc. of 0.1 % amylase were added to all systems. Half of the systems were stirred before addition of amylase, so as to break up the gel, which was left intact in the remainder. The starch content of all systems was determined after

standing for 1 hr. at 16°; the results, given in Table IV, show that considerable retardation (about 70 %) results from occlusion of starch within the gel, and that this retardation was considerably smaller (about 40 %) in those systems in which the gel had previously been broken up.

Table IV. *Effect of gelation of starch solution on velocity of reaction.*

Time of gelation hrs.	mg. reducing sugar found					
	Systems not stirred before addition of amylase			Systems stirred before addition of amylase		
	Starch added to gel	Starch and gelatin solutions mixed before gelation	% inhibition	Starch added to gel	Starch and gelatin solutions mixed before gelation	% inhibition
24	26.6	4.9	81.2	26.3	15.4	45.2
8	28	4.3	84.6	28.3	15.6	44.8
2	25	12	52	29.3	17.3	40.9
1	25.3	7.2	71.1	28	19.6	30.0

Similar experiments were next performed on occlusion of amylase by gelatin gels. Solutions containing 15 cc. of 3 % gelatin and 1 cc. of 0.1 % amylase or water were left for 24 hrs. at 16°, after which 10 cc. of 1 % starch solution or water were added; as in the previous experiment, the gel in half of the flasks was broken up by stirring before the addition of starch. The reducing sugar content was determined after standing for 90 min. at 16°, and amounted to 46 mg. in the control systems, to 7.6 mg. in the stirred systems, and to zero in those systems consisting of unbroken gel and amylase solution.

The velocity of reaction in gels containing both starch and amylase together was studied in the following way. A series of flasks was prepared; each contained 10 cc. of 1 % starch, 15 cc. of 0.5 or 5 % gelatin, and 1 cc. of 0.1 % amylase and was immersed in ice-water for 5 min., when the 5 % gelatin solutions set to a jelly. The flasks were then left in water at 16° for 4 hrs., after which reducing sugars were determined using Bertrand's method. 54 mg. of sugar were found in 0.5 % gelatin solutions, and 47 mg. in 5 % gelatin gels (5 experiments). This result points to the absence of retardation of reaction to any considerable extent by conversion of the medium from the liquid state to a jelly; the possibility existed, however, that this effect was only apparent, and that the reaction proceeded with great velocity in its initial phases, before gelation was complete. That this was not the case was shown by experiments in which reducing sugars were determined immediately on gelation, *i.e.*, 10 min. and 150 min. after addition of amylase, 1.8 and 40 mg. being found respectively (5 experiments).

(d) *Velocity of reaction in gels.* The experiments described above indicate that the velocity of reaction is chiefly influenced by the physical state (sol or gel) of one part of the system at the moment of addition of the other. The influence of mechanical breaking up of the gel was examined in the following way.

A number of systems containing 10 cc. of 1 % starch and 15 cc. of 2 % gelatin were allowed to stand for 20 hrs., during which time all systems set to a jelly. Two flasks were then shaken for 210 min., and 1 cc. of 0.1 % amylase was added, whilst amylase was immediately added to two other flasks, which were then shaken for 210 min. Amylase was also added to a third pair of flasks, which were not shaken at all and to the control systems not containing gelatin. Reducing sugar was determined in all systems, using Bertrand's method, 19.3 mg. being found in systems shaken before the addition of gelatin, 19.0 in those shaken after, 0.0 in unshaken systems, and 24 mg. in the control systems, both shaken and unshaken.

A number of similar systems were now set up, with the difference that gelation was not allowed to take place. Half the solutions were shaken for 2 hrs., and all flasks were then left for 20 hrs. at 30°, after which 1 cc. of 0.1 % amylase was added to all flasks, and reducing sugar was determined after 1 hr. at 30°. Practically the same values were found in all systems, *viz.* 46.8 and 46.0 mg. in shaken and unshaken gelatin solutions respectively, and 44 and 46 mg. in shaken and unshaken control solutions respectively.

(e) *Effect on amylolysis of inhibition of gelation due to addition of alcohol.* A number of systems were prepared, containing 10 cc. of 1 % starch, 5 cc. of 9 % gelatin solution or phosphate buffers at the same p_H , and 4 cc. of water or 96 % ethyl alcohol. Gelation is to a large extent inhibited by addition of alcohol. 1 cc. of 0.1 % amylase was added to all systems, and reducing sugar was determined after 4 hrs. at 17° and after 1 hr. at 30°. At 17° the control flasks without alcohol contained 59.7 mg., whilst with alcohol 42.7 mg. of sugar were found, indicating a certain inactivation of amylase by alcohol. In systems containing gelatin, those to which alcohol had not been added contained 5.3 mg., whilst in the presence of alcohol 40.2 mg. were found. At 30° gelation did not take place, and alcohol has consequently an exclusively inactivating action, 44 and 31.4 mg. of reducing sugar respectively being found in control systems without and with alcohol, and 44.9 and 32.6 mg. in the corresponding systems with gelatin.

II. ACTION OF UREASE IN MEDIA CONTAINING GELATIN.

A number of systems were set up containing 10 cc. of 1 % urea, 5 cc. of phosphate buffer (p_H 5.9), 5 cc. of water or gelatin of the requisite concentration and 1 cc. of 0.1 % urease. The flasks were left for 1 hr. at 30°, after which ammonia was determined by Folin's aeration method. The velocity of reaction was the same in all cases, 66–68 mg. of urea being found in all systems containing 0.8 % of gelatin.

DISCUSSION.

The velocity of diffusion is, as follows from Einstein and Smoluchowski's formula, inversely proportional to the viscosity of the medium, whence it might be expected that in certain heterogeneous systems increase in viscosity

would lead to retardation of reaction. In the case of the system amylase-0.3 % starch-gelatin, however, no retarding effect is observed below 5 % gelatin, in spite of the considerable viscosity of such systems, whilst using 0.6 % starch retardation of reaction is inconsiderable even in 10 % gelatin. This difference might be ascribed to the relatively sparser distribution of starch particles in the former case than in the latter, as a consequence of which the average distance travelled through the solution before collision with amylase particles is greater; since the time required to travel this distance is proportional to the viscosity of the medium, factors connected with diffusion have a greater influence in more dilute than in more concentrated solutions. The above results are not in agreement with the experiments described in Part VIII of this series, in which 1 % gelatin exerted a considerable inhibitory action on similar systems; the p_H of the systems studied earlier was, however, different from that maintained in the above reactions, and hence the apparent discrepancy.

In the case of the system urease-urea-gelatin, the velocity of reaction appears to be independent of the viscosity of the medium.

The experiments on jellies indicate that diffusion of colloidal particles such as starch or amylase is practically nil, *i.e.* that on the addition of a solution of one substrate to a jelly containing the other practically no reaction takes place unless the jelly is broken up. Where both enzyme and substrate are enclosed within the same jelly, the reaction velocity is little inferior to that in solutions. It is possible approximately to calculate spatial separation of the individual amylase particles; this calculation can be, of course, only relatively correct as to the order of magnitude. The systems in question contained 100 mg. of starch and 10 mg. of amylase in 16 cc. of jelly. Assuming that the given sample of amylase contains 1 % of pure enzyme, and that the mol. wt. of the latter is about 20,000, it follows that about 2×10^{16} molecules are present in the system. Taking the mol. wt. of starch to be 40,000, there should at the same time be present 10^{20} starch molecules, whence it follows that the system contains 5000 starch molecules per molecule of amylase, and that the average distance between individual amylase molecules is about $100\mu\mu$. According to Hardy [1928] the sphere of action of colloidal particles may exceed $100\mu\mu$, whence it would follow that the immobilisation of the above system need not necessarily inhibit reaction. An alternative explanation would be that amylase particles combine immediately with the entire number of starch molecules corresponding to one enzyme particle, and that subsequent gelation of the system would hence be without effect on the velocity of reaction. This explanation would, however, be incompatible with Michaelis and Menten's [1913] theory of formation of enzyme-substrate compounds, and with the values obtained for the dissociation constant of this compound. A third possible explanation would be the possibility of local thixotropic [Freundlich, 1928] liquefaction of the jelly in the vicinity of amylase particles, with consequent mobilisation of the enclosed starch.

Finally, the possibility exists that gelatin jelly is not a homogeneous mass, but that it contains lacunae filled with more liquid substance, and that amylase and starch are chiefly concentrated in these spaces. Should both amylase and starch be present in these spaces, they would be able to move in the same way as in gelatin solution, so that the reaction could proceed with considerable velocity. The next part of this series will be devoted to a closer investigation of this phenomenon.

SUMMARY.

1. The quantity of sugar found by Bertrand's method in the presence of gelatin is smaller than in its absence. The apparent diminution in sugar content increases with concentration of gelatin.

2. The velocity of reaction in the system amylase-0.6 % starch-gelatin sol is unaffected below 9 % gelatin, and appears to be little dependent on the viscosity of the medium. Where 0.3 % starch is taken, no retardation of reaction is observed below 5 % gelatin, whilst at higher concentrations of gelatin increasingly great retardation of reaction takes place.

3. The velocity of the reaction between urease and urea is the same in the presence or absence of gelatin.

4. The rate of diffusion of starch and amylase in gelatin jellies is considerably smaller than in similar sols; as a consequence the velocity of reaction in systems containing one substrate in solution and the other enclosed in a jelly is very small. It may be greatly accelerated by destruction of structure, either mechanically or by the addition of alcohol.

5. The velocity of reaction between amylase and starch in gelatin solution is little greater than in gelatin jelly.

REFERENCES.

- Freundlich (1928). *Kolloid Z.* **46**, 289.
Hardy (1928). *Kolloid Z.* **46**, 268.
Michaelis and Menten (1913). *Biochem. Z.* **49**, 333.
Przyłęcki and Gurfinkel (1930). *Biochem. J.* **24**, 179.

LXXIX. EFFECT OF ADDITION OF SALTS ON THE ISOELECTRIC POINT OF PROTEINS. I

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THE question whether the isoelectric point (I.P.) of proteins is a fixed, invariable value or not is of great importance in the study of equilibria in biological systems, as a consequence of the fact that the physical state, osmotic pressure, viscosity, coagulation, gelation, imbibition, sorption of other colloids, *etc.* of colloidal solutions depend on how far they are from the I.P., *i.e.* on the value of the charge present on the surface of the colloidal particles.

If it be accepted that, according to Hardy [1899] and Michaelis [1923], the colloidal particles are discharged at the I.P., *i.e.* that no potential difference exists between the disperse and the dispersed phases, then the normality of anions and cations should be equal ($[a'] = [a'']$) for ampholytes at the I.P. The I.P. would thus depend, apart from the nature of the colloid, on the presence of various substances in the system; those substances which can affect the ratio $[a'']/[a']$ must alter the p_H of the I.P.

Michaelis [1923] has expressed the opinion that the p_H of the I.P. is invariable. On the other hand, it has been shown [Gerngross and Bach, 1923; Küntzel, 1929; Ostwald *et al.*, 1925; Pauli and Valko, 1929; Wilson, 1922], that the p_H of protein solutions at the I.P. may vary considerably according to the state of purity of the protein, or to the nature and concentration of salts present; the mechanism of action of mineral salts would appear to depend on inter-ionic forces and on the formation of complex salts. The explanation of the action of salts is simpler in the case of lyophobes than of lyophils; in the former case the degree of dispersion and the stability of the sol are functions solely of the charge of the particles, whilst in the latter case two factors may exist, *viz.* the affinity of certain undissociated parts of the colloid (Loeb's hydrophilic groups) for the medium, in addition to the charge of the ionised particles. The difference between lyophils and lyophobes lies therefore chiefly in the nature of the forces affecting affinity for the medium, whilst those factors which are connected with the charge are, apart from its actual origin, probably the same in both cases. The theoretical aspect of the question of the variability of the I.P. is thus the same for both types of colloid, and for this reason the same factors should in both cases affect the p_H of the I.P.

The action of salts on the charge of lyophobic colloidal particles may

depend, in the case of anti-ions (Pauli's Gegenionen), on the concentration and the valency of the anti-ion, on the extent to which it is able to polarise the colloidal surface, on the dimensions, *i.e.* the degree of hydration of the anti-ion, on its heteropolar structure, and on changes in hydration, and hence of solubility, of the product of association; in the case of iso-ions (Pauli's Nebenionen) on the activity factor of the salt (thus the anti-ion K^+ has a different activity according to the anion with which it is associated), on the formation of difficultly soluble salts and complexes, on the increase in charge of the colloidal particles, due either to the partial neutralisation of one of the charges of ampholytes, or to adsorption of iso-ions, or, finally, to changes in the lyophilic or lyophobic character of the colloid.

It is our opinion that the above postulates may, except for high concentrations of salt, be applied also to lyophilic colloids. Experiments will be described in this paper in which the action of salts on isostable types of protein, such as gelatin and serum-albumin, and on isolabile caseinogen, is studied under various conditions.

EXPERIMENTAL.

The experimental material taken was crystallised serum-albumin (Merck), purified caseinogen, and purified gelatin [Przyłęcki, 1930]. These substances were dissolved in such amounts that the final concentrations in the systems studied, after addition of all reagents, were 0.25, 0.5, 1.0 and 2.0 %. The p_H of the systems was adjusted by adding the requisite amount of 0.02 *N*, 0.1 *N* or *N* sodium hydroxide or hydrochloric acid. p_H was determined potentiometrically, using a hydrogen electrode; in certain cases it was determined before and after addition of salt, and before and after boiling. The salts used were Merck's chemically pure reagents and were added before boiling or addition of alcohol to the systems.

The action of heat was studied in the following way. Solutions of different salts were placed in a number of test-tubes of approximately equal diameter, and an equal volume of water was placed in the control test-tubes. Water was added to 5 cc. in all test-tubes, 10 cc. of serum-albumin solution were added, and the contents of all tubes were thoroughly mixed. The test-tubes were then placed in a holder, which was immersed in boiling water and left for 10 or 20 min. before removal of the systems from the water-bath. In some cases the degree of coagulation was roughly determined by filtering and comparing the intensity of the biuret coloration given by the filtrates.

The effect given by salts at laboratory temperature was studied on acid and alkaline solutions of caseinogen, prepared respectively by dissolving caseinogen in 0.03 *N* hydrochloric acid and 0.02 *N* sodium hydroxide. Different concentrations of various salts were added to 1 % solutions of this protein, and the production of opalescence, turbidity, or precipitation was noted.

The experiments performed in the presence of alcohol were carried out

in tall 200 cc. cylinders, in which 10 cc. of serum-albumin, caseinogen or gelatin solutions were placed, and 2 cc. of salt solution and water were added to all systems. Equal volumes of 90 % alcohol were then successively added to all systems, the final alcohol concentration of which was 85 %. The molar concentration of the salts present was for these systems calculated for 12 cc. of aqueous solution, without taking the volume of added alcohol into account.

A. SERUM-ALBUMIN.

Effect of boiling in acid solutions.

The dependence of critical concentration (C_K) on the valency of iso- and anti-ions and on p_H was examined in the following way. Hydrochloric acid was added to 1 and 1.5 % solutions of serum-albumin (this will in future be designated as S.A.), to give a series of p_H 4.2, 3.9, 3.2, 2.6, 2.2, 1.4 and less than 1, and all systems were placed in boiling water. Coagulation was observed at p_H 4.5, and opalescence at p_H 4.2–3.9, clear solutions at p_H 3.2–1.4; at $p_H < 1$ the solutions again became turbid, and in 0.5–0.7 *M* hydrochloric acid coagulation again occurred.

Table I.

(C_K for 1 %, serum-albumin heated at 100°).

Salt	p_H 4.1 concentration		p_H 3.2 concentration		p_H 2.2 concentration	
	Molar	Normal	Molar	Normal	Molar	Normal
$K_3Fe(CN)_6$	< 0.00005	< 0.00015	0.00016	0.0005	0.0007	0.0021
Na_2SO_4	0.0024	0.0048	0.0094	0.0188	0.020	0.040
$MgSO_4$	0.0072	0.0144	0.031	0.062	0.150	0.300
$ZnSO_4$	—	—	0.045	0.090	—	—
$CuSO_4$	—	—	0.0576	0.1132	—	—
$\frac{1}{3}Fe_2(SO_4)_3$	0.017	0.034	0.060	0.120	—	—
$MgCl_2$	0.056	0.112	0.200	0.400	0.390	0.780
$CaCl_2$	0.053	0.106	0.180	0.360	—	—
$ZnCl_2$	0.110	0.220	0.270	0.540	—	—
$NaCl$	0.074	0.074	0.280	0.280	0.560	0.560
KCl	0.068	0.068	—	—	—	—

The addition of salts provoked coagulation at certain definite concentrations (C_K), which varied according to the p_H of the solution, being greater the further this was removed from the i.p. (cf. Table I).

Table I a.

Ratios of C_K of various salts.

Salts	p_H 4.1	p_H 3.2	p_H 2.2
$Na_2SO_4/MgSO_4$	1/ 3.0	1/ 3.3	1/ 7.5
$Na_2SO_4/ZnSO_4$	—	1/ 4.7	—
$MgSO_4/ZnSO_4$	—	1/ 1.45	—
$Na_2SO_4/CuSO_4$	—	1/ 6.1	—
$MgSO_4/MgCl_2$	1/ 8	1/ 6.5	1/ 2.6
$Na_2SO_4/NaCl$	1/16.25	1/14.9	1/14.0
$NaCl/MgCl_2$	1/ 1.5	1/ 1.4	1/ 1.4
$MgCl_2/ZnCl_2$	1/ 2.0	1/ 1.35	—
$Na_2SO_4/MgCl_2$	1/23	1/21.3	1/19.5

In Table II are given the results expressing the relation between the p_H of the solutions, the concentration of hydrochloric acid and the C_K of sodium sulphate. It will be seen that the concentrations of acid and salt are directly proportional to one another, whilst such a relation between p_H and C_K holds only over a narrow range. In strongly acid solutions (0.5–0.7 *N*) coagulation takes place with or without the addition of salts, but in 0.4 *N* HCl coagulation occurs on the addition of very small quantities of salts ($MgCl_2$, NaCl, or Na_2SO_4). The curves connecting C_K of salt with p_H of solution exhibit a maximum at p_H 1.4–2.2.

Table II.

C_K of Na_2SO_4 at various p_H and concentrations of hydrochloric acid.

Conc. of HCl <i>M</i>	0.009	0.0167	0.0227	0.0286	0.050	0.500
p_H	3.99	3.22	2.60	2.20	1.40	1
C_K Na_2SO_4 (<i>M</i>)	0.0046	0.0114	0.017	0.0205	0.040	0.003
$[HCl]/[Na_2SO_4]$	2.0	1.5	1.3	1.4	1.25	170.0

The salts studied can be classified into a number of groups, according to the valency of the anion; thus potassium ferricyanide in concentrations of less than 0.001 *M* produces coagulation at 16°, but is slightly less active at 100°, whilst the bivalent sulphate ion has a much weaker action, and using chlorides (NaCl, $MgCl_2$, $CaCl_2$) C_K is so high that the dehydration factor has to be taken into account in addition to the ionic effect. The following series of C_K is obtained for ter-, bi-, and univalent anions:

p_H 4.1	$Fe(CN)_6''' : SO_4'' : Cl' = 1 : 32 : 520$
p_H 3.2	1 : 31.3 : 470
p_H 2.2	1 : 20 : 350
p_H 4.1	$SO_4'' : Cl' = 1 : 16.3$
p_H 3.2	1 : 14.9
p_H 2.2	1 : 14.0

Apart from the above relations, which are in agreement with Hardy's and Schulze's anti-ion valency rule, a very interesting relation between the nature of the iso-ion and the C_K of the anti-ion is apparent. If we compare the value of C_K for sodium, magnesium, and ferric sulphates we see that at p_H 4.1, for example, it is lowest for sodium sulphate (0.0024 *M*), three times as great for magnesium sulphate (0.0072 *M*), and over seven times as great for ferric sulphate (0.034 *M*). For solutions the p_H of which is further removed from the i.p. these differences increase, until at p_H 2.2 we have the ratio $Na_2SO_4 : MgSO_4 = 1 : 7.5$. The same phenomenon holds, although to a less marked extent, for salts possessing univalent anions; thus at the same p_H C_K is 1.4 times as great for magnesium as for sodium chloride. The action of bivalent cations was further studied using copper and zinc sulphates, and zinc and cadmium chlorides. The results obtained with zinc and copper salts are of the same order as those obtained using alkaline earth salts, whilst cadmium chloride has a considerably higher C_K than have other salts possessing a bivalent cation.

Only very small changes in p_H are noticed as a result of coagulation of

protein. Thus, in one case, the p_H of a control system containing serum albumin, acid and water was 3.02 both before and after boiling, and the p_H of the same system with sodium sulphate was 3.26 before boiling, and 3.30 after.

Table III.

Influence of dilution of albumin on the C_K of sodium sulphate.

% albumin C_K	2	1	0.5	0.25
	0.0094 <i>M</i>	0.0078 <i>M</i>	0.0062 <i>M</i>	0.0054 <i>M</i>

If coagulation is brought about by the presence of definite concentrations of salt, dilution of the whole system before boiling should inhibit coagulation. This was found to be the case; the addition of an equal volume of water at the same p_H as that of a system in which coagulation occurred after heating for a few minutes at 100° prevented coagulation. In connection with these experiments, it was noticed that the C_K of salts diminishes with the dilution of albumin (Table III).

Dependence of C_K for sodium sulphate on the peptising acid.

The results given in Table IV show that the C_K of sodium sulphate is considerably higher for hydrochloric acid than for sulphuric acid solutions of albumin (0.020 and 0.012 *M* respectively). This difference is due partly to the coagulating effect of the sulphate ions originating from the sulphuric acid originally present in the second case: it is, however, of interest that the total sulphate ion concentration is greater at coagulation for such systems than for hydrochloric acid solutions, and the difference between the action of the SO_4 from sulphuric acid and from sodium sulphate increases with greater original concentration of sulphuric acid (Table IV).

Table IV.

C_K of sodium sulphate in acidified systems, containing 50 cc. of 1% serum-albumin and 30 cc. of 0.1 *N* acid.

Solutions are	<i>M</i> conc. of Na_2SO_4 in the presence of	
	H_2SO_4	HCl
Clear	0 -0.005	0 -0.0142
Opalescent	0.005 -0.012	0.0142-0.023
Coagulated	Over 0.012	Over 0.023

Simultaneous action of different anti- and iso-ions.

The addition of sodium sulphate up to a definite concentration to solutions of serum-albumin in the presence of sub-critical concentrations of potassium ferricyanide leads to coagulation, even in unheated solutions. This effect is due to summation of the action of different multivalent anti-ions, and the C_K of the second salt in the presence of the first is greater, the greater the dilution of the first below its C_K (Table V).

Table V.

Coagulative effect of Na_2SO_4 in the presence of sub-critical concentrations of ferricyanide at p_{H} 2.

M concentration of Na_2SO_4	No $\text{K}_3\text{Fe}(\text{CN})_6$	0.0004 M $\text{K}_3\text{Fe}(\text{CN})_6$	0.0006 M $\text{K}_3\text{Fe}(\text{CN})_6$
0	Clear	Clear	Slight opalescence
0.017	"	Opalescent	Flocculation
0.025	"	Intense opalescence	"
0.050	"	Flocculation	"

The addition of magnesium or aluminium chloride to systems containing sodium sulphate, keeping the p_{H} and volume constant, has a contrary effect, raising the C_{K} of the latter salt. The magnitude of this effect depends on the p_{H} of the systems, on the concentration of salt, and on the valency of the iso-ion (Table VI). Thus, adding various quantities of magnesium chloride

Table VI.

Peptising effect of various iso-ions in systems containing 0.030 M Na_2SO_4 at p_{H} 2.2.

Solutions are	M conc. of added salt			
	NaCl	MgCl_2	FeCl_3	AlCl_3
Flocculated	0.057-0.57	0 -0.042	0 -0.020	0 -0.010
Opalescent	—	0.042-0.083	0.020-0.035	0.010-0.021
Clear	—	Over 0.083	Over 0.035	Over 0.021

to a 1 % albumin solution at p_{H} 2.65, the maximum elevation of C_{K} for sodium sulphate occurs in 0.1 M MgCl_2 , whilst at p_{H} 3.6 the maximum C_{K} is in 0.05 M MgCl_2 . In general, the peptising action of magnesium chloride diminishes as the p_{H} of the solution differs more and more from the i.p. The action of aluminium chloride is considerably greater than is that of magnesium chloride, since the latter salt under optimal conditions increases the C_{K} of sodium sulphate 2.5 times, and the former 6.7 times (Table VII). At the same

Table VII.

Peptising action of MgCl_2 and AlCl_3 .

M conc. of MgCl_2	C_{K} of Na_2SO_4 at		M conc. of AlCl_3	C_{K} Na_2SO_4 at p_{H} 3.0
	p_{H} 3.6 (M)	p_{H} 2.65 (M)		
0	0.0064	0.0161	0	0.010
0.025	0.014	—	0.007	0.0175
0.050	0.020	0.031	0.010	0.022
0.100	0.0177	0.037	0.015	0.026
0.150	—	0.030	0.030	0.055
0.200	0.0155	—	0.055	0.067
0.250	—	0.024	—	—

time we see that the C_{K} of magnesium chloride is greater than that of aluminium chloride (0.08 and 0.05 M respectively, at p_{H} 3.2). Similar results for the peptising effect of these two salts were obtained for systems coagulated in the cold by the addition of ferricyanide (Table VIII).

Table VIII.

Peptising action of iso-ions in 2 % serum-albumin systems containing ferricyanide at p_H 2.0 and 20'.

<i>M</i> conc. of ferri- cyanide	Effect on solutions of addition of					
	H ₂ O	0.03 <i>M</i> HCl	0.3 <i>M</i> HCl	0.6 <i>M</i> NaCl	0.09 <i>M</i> MgCl ₂	0.05 <i>M</i> AlCl ₃
0.0004	Clear	Clear	Opalescence	Opalescence	Clear	Clear
0.0008	Opalescence	Opalescence	Turbidity	Turbidity	"	"
0.0012	Flocculation	Flocculation	Flocculation	Flocculation	Opalescence	Opalescence
0.0016	"	"	"	"	"	"
0.0020	"	"	"	"	Turbidity, then floccu- lation	"
0.0024	"	"	"	"	Flocculation	Flocculation
0.0030	"	"	"	"	"	"

Effect of boiling in alkaline solution.

The action of the salts NaCl, MgCl₂, CaCl₂, MgSO₄, AlCl₃ and FeCl₃ on serum-albumin solutions at p_H 9.2 was examined. Aluminium and ferric chlorides in higher concentrations were to a small extent precipitated by alkaline solutions. Normal sodium chloride did not coagulate alkaline albumin even on boiling, whilst coagulation took place in 0.026–0.027 *M* calcium or magnesium chlorides without heating. The C_K of magnesium sulphate is 0.033 *M*, *i.e.* 27 % greater than for calcium chloride. The C_K of aluminium and ferric chlorides could not be determined with any accuracy, and we do not attach any special significance to our figures (Table IX).

Table IX.

C_K of various salts for 1 % serum-albumin solutions at p_H 9.2.

NaCl	CaCl ₂	MgCl ₂	MgSO ₄	AlCl ₃	FeCl ₃
0.640 <i>M</i> (opalescence)	0.026 <i>M</i>	0.027 <i>M</i>	0.033 <i>M</i>	0.0045 <i>M</i>	0.006 <i>M</i>

The C_K of magnesium chloride was determined alone and in the presence of sodium chloride or sulphate and potassium ferricyanide. All systems were at p_H 9.2, and contained 1 % serum-albumin, and 0.03 *M* MgCl₂, and were, in the absence of other salts, in a state of flocculation. The addition of 0.014 *M* Na₂SO₄ or of 0.0016 *M* K₃Fe(CN)₆ prevented flocculation; the systems exhibited, however, intense opalescence. As the concentration of salts is augmented, the solutions become increasingly clear, so that in the presence of 0.03 *M* Na₂SO₄ or 0.003 *M* K₃Fe(CN)₆ they are practically quite clear (Table X).

Table X.

C_K for MgCl₂ at p_H 8.5 (*M*).

C_K MgCl ₂	MgCl ₂ alone	In presence of NaCl			In presence of Na ₂ SO ₄		
		0.15	0.30	0.55	0.0135	0.020	0.025
	0.025	0.0263	0.0257	0.022	0.0335	0.035	0.032

Precipitation by alcohol.

The relation between the C_K of sodium sulphate, the p_H of the system, and the concentration of hydrochloric acid was determined in the presence of 85 % ethyl alcohol. The results, given in Table XI, show that a proportionality exists between the concentration of acid and the C_K of sodium sulphate.

Table XI.

Comparison of C_K of Na_2SO_4 for boiled systems and for 85 % alcoholic solutions.

p_H	C_K for systems heated at 100°	C_K for 85 % alcohol systems	C_K 100°	$[\text{Na}_2\text{SO}_4]/[\text{HCl}]$	
	(M)	(M)	C_K alcohol	100°	Alcohol
3.2	0.0096	0.0016	6.0 : 1	1.6	9.6
2.6	0.0145	0.0027	6.1 : 1	1.5	10.0
2.0	0.0223	0.0042	5.3 : 1	1.75	11.0
1.6	0.031	0.0057	5.5 : 1	1.6	10.0
1.4	0.042	0.009	4.7 : 1	1.5	8.4

Comparison between the C_K of sodium sulphate in systems coagulated by heat and by alcohol.

C_K was determined for sodium sulphate in acid serum-albumin solutions, in the presence of 85 % alcohol, and heated at 100°. The value of C_K is at p_H 3.2 six times greater in the latter case than in the former, but the difference diminishes as more acid solutions are taken, so that at p_H 1.6 the ratio is 5:1. Acidification of the solutions to a certain extent augments C_K for both systems.

B. CASEINOGEN.

C_K was determined for sodium sulphate and potassium ferrieyanide in caseinogen solutions at p_H 2, in unheated and heated systems and in the presence of 85 % alcohol. The results, given in Table XII, show that C_K is 0.0076 M in unheated systems, 0.0064 M at 100°, and 0.005 M in the presence of alcohol. The ratio of the C_K values in heated and alcoholic systems is 1.27, in unheated and heated systems 1.19, and in unheated and alcoholic solutions 1.52; these ratios differ considerably from those obtained for serum-albumin.

A second series of experiments on the relation between p_H and C_K showed that the C_K of sodium sulphate increases with the charge on the colloidal particles.

Table XII.

C_K of sulphate ions for caseinogen solutions acidified with hydrochloric acid to p_H 2 (M).

At 15°	Heated 10 min. at 100°	In 85 % alcohol
0.0076	0.0064	0.005

Determination of the C_K of sulphate ion added as a mixture of hydrochloric and sulphuric acids and of hydrochloric acid and sodium sulphate showed that in the former case it has a value of over 0.08 M, whilst in the latter it is 0.0067 M.

C. GELATIN.

Systems heated at 100°.

The addition of salts giving rise to univalent or bivalent ions (0.001–0.2 *M*) to gelatin solutions at p_H 1–12 does not produce coagulation, either in heated or unheated systems. Potassium ferricyanide coagulates in 0.0005–0.001 *M* solutions, according to the p_H , whilst ferrocyanide is without action.

Action of salts in the presence of alcohol.

The results for systems containing 80 % alcohol (Table XIII) are in every way similar to those obtained for serum-albumin. The C_K of sodium sulphate in systems containing 0.5 % gelatin and 50–90 % alcohol falls at first rapidly with increasing concentration of alcohol, from 0.08 *M* in 50 % to 0.0008 *M* in 85 % alcohol, above which concentration no further change is observed (Table XIV). The value of C_K rises with increasing acidity from 0.00045 *M* in 0.005 *M* HCl to 0.009 *M* in 0.06 *M* HCl (Table XV).

Table XIII.

C_K of salts in gelatin solutions containing 80 % alcohol (dilution by alcohol taken into consideration) (*M*).

p_H 7.30			p_H 1.75			
KCl	MgCl ₂	AlCl ₃	NaCl	Na ₂ SO ₄	MgSO ₄	K ₃ Fe(CN) ₆
0.017	0.0025	0.00025	Over 0.15	0.003	0.0014	0.00015

Table XIV.

Relation between C_K and concentration of alcohol.

C_K of Na ₂ SO ₄ (<i>M</i>)	% alcohol	C_K of MgSO ₄ (<i>M</i>)	% alcohol
0.0008	85	0.011	71
0.0016	80	0.014	69
0.0032	71	0.016	66
0.0047	66	0.020	62
0.0064	62		
0.008	59		
0.032	55		
0.040	54		
0.080	52		

Table XV.

C_K of Na₂SO₄ at various p_H .

p_H	C_K of Na ₂ SO ₄ (<i>M</i>)
3.8	0.00045
3.0	0.0012
2.5	0.0021
2.2	0.0027
1.5	0.009

Action of iso-ions.

The action of iso-ions is illustrated by experiments in which the C_K values of sodium and magnesium sulphates in the presence of alcohol were compared.

In 66 % alcohol the value of C_K was 0.0047 M for sodium, and 0.016 M for magnesium sulphate, whilst in 71 % alcohol the corresponding values were 0.0032 M and 0.011 M (Table XIV).

Table XVI.

Peptising action of $MgCl_2$ in various concentrations of alcohol.

Salts present	Concentration of alcohol necessary for flocculation %
0.0047 M Na_2SO_4	66.5
0.008 M Na_2SO_4	60.0
0.008 M Na_2SO_4 + 0.1 M $MgCl_2$	73.0
0.016 M Na_2SO_4 + 0.1 M $MgCl_2$	70.5
0.024 M Na_2SO_4 + 0.1 M $MgCl_2$	69.5
0.032 M Na_2SO_4 + 0.1 M $MgCl_2$	66.5

When magnesium chloride is added in addition to sulphate, the C_K of the latter rises, from 0.011 M in 71 % alcohol to 0.016 M in the presence of 0.05 M $MgCl_2$ (Table XVI); this effect may be ascribed to the more powerful peptising effect of the magnesium iso-ion in the presence of the feebly coagulating chloride ion. The peptising effect of magnesium ions is not evident in systems containing over 80 % of alcohol; thus in 83 % alcohol 0.002–0.003 M $MgSO_4$ coagulates gelatin, and the addition of magnesium chloride (0.01–0.05 M) does not affect the results obtained.

Similar results were obtained for systems containing sodium sulphate, magnesium chloride and alcohol. The C_K of sodium sulphate is greatly increased by the presence of 0.1 M $MgCl_2$ in low concentrations of alcohol, but less so in higher concentrations; thus in 66 % alcohol C_K is raised from 0.0044 to 0.032 M , whilst in 71 % alcohol the corresponding values are 0.0032 and 0.007 M , and in 85 % alcohol peptisation due to magnesium ions is entirely absent, only the feebly coagulating effect of chloride ions being superimposed on that of sulphate ions.

DISCUSSION.

The stability of protein solutions depends on two factors—the affinity to water of the undissociated part of the protein molecule, and the charge of the colloidal particles. Proteins have been classified by Wo. Ostwald [1920] into isostable and isolabile; at the I.P. the affinity of the first for water exceeds their critical hydration value [Kruyt and de Jong, 1928], so that stable solutions of such proteins may be obtained without the addition of peptisers. Isostable proteins may be further classified into thermo-isostable and thermo-isolabile, according to the behaviour of their solutions on heating; should the affinity for water fall below the critical value at higher temperatures thermo-isolabile proteins coagulate. The affinity to water of isolabile proteins is even at the ordinary temperature less than the critical, so that the stability of sols of such proteins is a function of the charge on the particles. No sharp division exists between isostable and isolabile proteins; the thermo-isolabile group

represents a transition form between the two groups and certain of the thermo-isolabile proteins may at the i.p. be considered to be in a state of slow coagulation.

Isostable proteins may be obtained in the isolabile state, either by heating at above the critical temperature, in the case of thermo-isolabile proteins, or by the addition of such organic solvents as alcohol, acetone, ether, chloroform, *etc.* In the case of systems rendered isolabile by the addition of alcohol, two interesting observations, evidencing the relationship between the critical concentration of alcohol and the affinity of the given protein for water, are to be noted. These are the behaviour of purified protein at the i.p., and the difference between the action of electrolytes on protein solutions at p_H values other than the i.p. of the given protein and at the same p_H in the presence of alcohol. At the i.p. the C_K of alcohol increases with the isostability of the given protein, so that, *e.g.*, gelatin at the i.p. is coagulated by about 48 % alcohol, whilst serum-albumin is coagulated by 36 % alcohol.

At p_H values other than that of the i.p. the properties of various proteins with respect to the behaviour of their aqueous solutions on the addition of salts approach more nearly to one another as the concentration of alcohol in the system rises. Thus at p_H 2.5 aqueous gelatin is precipitated by 0.6*M*, serum-albumin by 0.5*M*, and caseinogen by 0.0076*M* Na_2SO_4 . In heated solutions, the corresponding values of C_K are respectively 0.55, 0.031 and 0.0064*M*; here the C_K of sodium sulphate for serum-albumin is very close to that for caseinogen. Finally, in 85 % alcohol, the values of C_K are respectively 0.006, 0.0057 and 0.005*M*, showing that in these conditions the properties of these apparently so different proteins have approached closely to one another.

As the concentration of alcohol rises, the charge on the colloidal particles has to be increased in order to maintain solution. Coagulation may be effected at any p_H using the same concentration of alcohol as at the i.p., by adding a critical concentration of electrolyte. As the concentration of alcohol rises, dehydration increases, with consequent diminution in C_K of the electrolyte, which varies from 0.08*M* in 50 % to 0.0018*M* in 90 % alcohol. This phenomenon might be interpreted variously. Thus it might be supposed that even in high concentrations of alcohol gelatin still possesses a certain affinity for water, and that hydration remains as one of the stabilising factors. The stabilising effect of hydration may, however, be in itself insufficient to prevent coagulation without the additional action of a charge on the particles, the neutralisation of which would therefore lead to coagulation. In connection with this theory it would be necessary to assume that the particles are not completely discharged at the C_K , and that, as a result of a certain residual affinity for water, stabilisation is the resultant of the summation of two factors. For this reason the greater the degree of dehydration, the more the C_K of sodium sulphate approaches that for systems the stabilisation of which depends exclusively on the charge. Various other explanations might be put

forward; in the present state of knowledge of the subject, however, these would be somewhat speculative. These explanations would include possible variations in the thickness of the electrical double layer, decrease in the charge, changes in the dielectric constant and in the activity factor of the salts, and augmented association.

The observations recorded in this paper indicate that the dispersion of proteins in colloidal solution is due to two factors, which have to be reduced to a certain minimum for coagulation to supervene. This view is based on the following grounds. For isolabile proteins their charge is the decisive factor for the stabilisation of the sol. Since the charge is a consequence of the structure of both isolabile and isostable proteins, there is no reason for supposing that the latter class are not charged in solution. The C_K of various salts for isolabile proteins are very similar in ordinary and in dehydrated solutions (boiled, or in the presence of alcohol); in such solutions the C_K for iso-labile and -stable proteins are very close to one another, suggesting that such factors as alcohol or heat convert isostable proteins into a form similar to the isolabile. This process depends either on the action of the factors applied on groups on which depends the affinity of the given protein for water, or on changes in the concentration or physical state of water (*e.g.* increased mobility at 100°). At the same time the nature of the factors to which the charge on the particles is due does not undergo change, with the exception of variation in the thickness of the double layer and in its composition, and for this reason the dehydrated form of isostable proteins is well adapted to the study of variations in the charge.

This depends on the ratio of anions to cations; when these are present in equivalent concentrations, the charge is zero. If it be accepted that the charge is connected with the f_a of the dissociated salt and with the degree of dissociation of the colloid, as well as, within certain p_H limits, with zwitterion dissociation, the action of ions will depend on the degree of association and the f_a of the salts produced. These factors are the same as those taking part in homogeneous systems; in protein solutions the charge on the particles is further affected by surface forces, and, above all, by the thickness of the double layer.

The observed facts might be given a different interpretation from that advanced above; it might be considered that ions, in particular anti-ions, form salts with protein ions, and that the solubility of certain of the salts thus formed is less than that of the free protein. The coagulative action of salts would then depend not on their action on the charge on the particles but on the formation of difficultly soluble salts. This explanation is, however, improbable, in view of the following facts. In a relatively dehydrated medium containing alcohol, the C_K of salts for different proteins have very similar values, in spite of the fact that the solubilities of these proteins and probably therefore of their salts are very different. Further counter-arguments are that the action of ions is connected with their valency, that iso-ions act in the

opposite way to anti-ions, that the closer the system is to the I.P., the smaller is C_K , and that the C_K of sulphate ions is at the same p_H 4–6 times greater when they are added as sulphuric acid than when added as sodium sulphate.

All these observations are in agreement with the hypothesis that the action of salts is due to the action of their ions on the charge of the protein particles. Thus the action of the anti-ions conforms to the rule of Hardy and Schulze whereby the action of discharging anti-ions is a function of their valency. In our case, the ratio of the action of uni-, bi-, and trivalent anions is as 1:30:500; these ratios are similar to those found [Pauli and Valkó, 1929] for lyophobic gold sol (1:30:250). The connection between the action of anti-ions and the charge on the colloidal particles is shown by the observation that C_K rises as the p_H of the solution diminishes below the I.P., *i.e.* as the charge increases. The difference between the actions of sulphuric acid and sodium sulphate is due to their different actions on the double layer. Thus sulphuric acid up to a certain concentration augments the positive charge of protein, owing to the action of its cations, whilst the action of sulphate is due almost exclusively to its anions. The action of iso-ions is complex, and depends on the degree of dehydration of the colloid; where this is incomplete iso-ions inhibit the coagulative action of anti-ions, to an extent depending on their valency. The action of uni-univalent salts appears to be due chiefly to displacement by univalent or bivalent iso-ions from acid albuminates, whilst where multivalent iso-ions are added to systems containing multivalent anti-ions the increase in the C_K of the latter is due partly to reactions of double decomposition, resulting in products of a lower f_a ; thus the addition of magnesium chloride to sodium sulphate yields a certain amount of magnesium sulphate, the f_a of which is considerably lower than that of sodium sulphate. A further effect is that of attraction by iso-ions of anti-ions from the hydro-sphere of the protein particles to the bulk of the solution, with consequent augmentation of the free charge of the protein complexes.

From the point of view of the zwitterion theory, the antagonistic effect of iso-ions in the coagulation of protein by addition of anti-ions may depend on the formation of salts of lower f_a between the carboxyl residues and the iso-ions, thereby augmenting the free positive charge due to ionisation of the amino-groups; this would apply to systems at the p_H of the I.P., or slightly below this p_H . This view is supported by Loeb's [1924] experiments, which showed that the free charge of denatured proteins is increased by the addition of salts yielding multivalent iso-ions and univalent anti-ions. According to Dorfmann [1930] multivalent iso-ions may further affect the degree of hydration of colloids, in this way inhibiting coagulation.

In systems dehydrated by the addition of alcohol the action of multivalent iso-ions diminishes with increasing alcohol content; no obvious explanation of this phenomenon is apparent.

It appears from the work of Loeb [1924, 1925] and of Kruyt *et al.* [1928, 1929], as well as from the results described in the present paper, that the chief

factor in the coagulative action of electrolytes on proteins is the reduction of the free charge on the colloidal particles below a certain critical minimum. A certain difference exists between proteins discharged at the isoelectric point and at other p_H , due to the fact that on the alkaline side of the I.P. the particles are discharged by and consequently associated with cations, and on the acid side by anions; the conception of the isoelectric state would apply only to purified proteins of zero free charge, whilst proteins discharged by electrolytes at other p_H values than that of the I.P. should rather be termed electrically neutral at the given p_H .

SUMMARY

1. Serum-albumin solutions coagulate on heating at 100° at p_H above 4.7 or below 1.
2. Various mineral salts produce coagulation at a certain critical concentration (C_K) for each p_H . The C_K is at a maximum at p_H about 2, and varies in accordance with the Hardy-Schulze rule. The ratios of C_K for ter-, bi-, and univalent anti-ions are as 1:30:500.
3. The value of the C_K of anti-ions also depends on the valency of the accompanying iso-ion.
4. The value of C_K of salts depends on the nature of the peptising acid; it is less in solutions acidified with acids giving rise to bivalent than to univalent anti-ions.
5. The coagulative action of two or more anti-ions at concentrations below the critical is additive.
6. Salts possessing a univalent anti-ion and a multivalent iso-ion exert in certain concentrations a peptising action.
7. Analogous results to the above are obtained at p_H values higher than those of the isoelectric point, at which cations are anti-ions.
8. Coagulation can be effected by the addition of salts to systems containing 85 % alcohol at any p_H . The Hardy-Schulze rule is followed in such systems, and the maximum values of C_K are at p_H 2.
9. The C_K of sodium sulphate for caseinogen hydrochloride at p_H 2 has practically the same value at 20° , 100° , and in 85 % alcohol systems (respectively 0.0076 M , 0.0064 M and 0.005 M).
10. The C_K of the sulphate ion added as sulphuric acid is considerably higher, *viz.* over 0.1 M .
11. Gelatin in 80 % alcohol can be flocculated at any p_H by critical concentrations of anti-ions, the values of C_K varying according to the Hardy-Schulze rule. The value of C_K varies inversely with the concentration of alcohol.
12. The C_K of sodium sulphate increases with the free charge on the gelatin particles up to a certain maximum, after which it again falls, as for serum-albumin.

13. The C_K of anti-ions increases with the valency of the iso-ions. The critical concentration of alcohol necessary to flocculate gelatin at a given concentration of sodium sulphate is higher in the presence of magnesium chloride than in its absence, and conversely, at a given concentration of alcohol, the C_K of sodium sulphate is considerably higher in the presence than in the absence of multivalent iso-ions.

14. The peptising action of multivalent iso-ions is not apparent in high concentrations (85 %) of alcohol, in which salts possessing a multivalent iso-ion and univalent anti-ions exert a coagulative action.

15. Isostable proteins can by heating at 100° or addition of alcohol be converted into proteins of the isolabile type. The C_K of sodium sulphate is at the same p_H practically the same for serum-albumin, gelatin, and caseinogen in presence of hydrochloric acid in 85 % alcohol.

16. Salts possessing multivalent anti-ions are able to discharge protein particles at any p_H . C_K being different at each p_H for each salt. The coagulum so obtained differs from that obtained at the isoelectric point.

17. The above observations may be readily explained if it be assumed that the state of dispersion of proteins is a function of their affinity for water and of the free charge on the particles; the reduction of the latter below a certain critical minimum results in conversion of protein sols into gels. Flocculation cannot be explained as being due to the formation of variously soluble salts.

REFERENCES.

- Dorfmann (1930). *Kolloid Z.* **52**, 66.
 Gerngross and Bach (1923). *Biochem. Z.* **143**, 533.
 Hardy (1899). *J. Physiol.* **24**, 288.
 Kruyt (1929). *Kolloid Beih.* **29**, 396, 432, 436, 454.
 — and de Jong (1928). *Kolloid Beih.* **28**, 1.
 — and Lier (1929). *Kolloid Beih.* **28**, 407.
 Kuntzel (1929). *Biochem. Z.* **209**, 326.
 Loeb (1924). *Les proteines* (Alcan, Paris).
 — (1925). *La théorie de phénomènes colloïdaux*.
 Michaelis (1923). *Wasserstoffionen Konzentration* (Berlin).
 Ostwald (1920). *Kleines Praktikum der Kolloidchemie* (Dresden).
 — Kuhn and Bohme (1925). *Kolloid Z.* **20**, 412.
 Pauli and Valkó (1929). *Elektrochemie der Kolloide* (Vienna).
 Przyłęcki (1930). *Compt. Rend. Soc. Biol.* **56**, 134.
 Wilson (1922). *J. Amer. Chem. Soc.* **44**, 288.

LXXX. THE PHOSPHATASE ACTIVITY OF TRANSPLANTS OF THE EPITHELIUM OF THE URINARY BLADDER TO THE ABDOMINAL WALL PRODUCING HETEROTOPIC OSSIFICATION.

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THE presence of an enzyme capable of hydrolysing certain phosphoric esters has been demonstrated in bone and ossifying cartilage by Robison [1923], who with his collaborators has presented evidence that it is an active agent in ossification.

In general, this evidence has been adduced from three lines of observations: (a) survey of the normal distribution of the enzyme; (b) study of calcification in bone and cartilage in experimental rickets; (c) tissue culture *in vitro* of cartilage and bone. Some of the more important observations may be briefly cited. Normally phosphatase was found to occur in the ossified portions of young human embryos and infants, while non-ossifying cartilage was in all cases devoid of it [Martland and Robison, 1924]. When severely rachitic bones were immersed in solutions of calcium hexosemonophosphate or glycerophosphate, deposition of calcium phosphate took place in the hypertrophic, but not in the small-celled, or articular cartilage [Robison and Soames, 1924, 1930]. The addition of very small amounts of a phosphoric ester to solutions of calcium salts and inorganic phosphate in concentrations otherwise inadequate for deposition of calcium phosphate in rachitic bone led readily to calcification [Robison and Soames, 1930; Robison, Macleod and Rosenheim, 1930]: desiccation for 24 hours or the addition of such protoplasmic poisons as chloroform, acetone or potassium cyanide inhibited calcification in inorganic solutions but did not destroy the enzyme, and calcification resulted in the presence of phosphoric ester [Robison, Macleod and Rosenheim, 1930]. In tissue culture of the embryonic femur and mandible of the chick, phosphatase could be demonstrated in large amounts when hypertrophic cartilage and bone were present; in the absence of these and in certain areas which do not normally ossify in these structures phosphatase was not present in detectable amounts [Fell and Robison, 1929, 1930].

¹ The surgical work herein reported was carried out in the Department of Physiology, University College, London.

An osteogenic effect has been demonstrated in the epithelium of the urinary bladder, ureter and kidney pelvis of the dog [Huggins, 1930, 1, 2; 1931]. When, for example, a portion of the bladder epithelium is transplanted to the connective tissue of the abdominal wall, ossification occurs regularly after 18 to 20 days in the connective tissue, provided the epithelium survives. The bone so formed has a constant relationship to the epithelium; the epithelium never ossifies. The bone gives the typical crystallographic appearance of true bone when examined spectroscopically by means of the X-rays [Roseberry, Hastings, and Morse, 1931].

The object of the present experiment was (a) to ascertain if phosphatase occurs normally in the urinary bladder epithelium or fibrous tissue of the abdominal wall and whether it is synthesised in the bone formed by transplantation of the former to the latter, and (b) to study the progress of its synthesis in relation to the ossification.

METHODS.

Surgical. Under aseptic precautions and ether anaesthesia the abdominal cavity was opened through a small lower mid-rectus incision, and the urinary bladder delivered through the wound. A preliminary incision was made in the dome of the bladder down to the mucosa, and a portion of the epithelium, approximately 2.5×2.5 cm., stripped from the muscular wall by blunt dissection and cut away. The wound in the dome of the bladder was closed with a fine silk suture, in two layers. The excised epithelium was transplanted to the top of the sheath of the rectus abdominis muscle, sewed in at four corners with a silk interrupted suture. The abdominal wall was closed, without drainage, with three silk sutures.

At operation small portions of the whole thickness of the bladder wall, the epithelium alone, the muscular wall of the bladder alone, and the connective tissue of the rectus sheath were excised for chemical examination as control specimens. The dogs were killed at intervals of 5, 9, 13, 16, 18 and 26 days after operation. At necropsy, many samples of tissue in the region of the transplant were removed and divided into nearly equal portions, the one half for histological examination and the other for phosphatase determinations.

Chemical. The method for determination of phosphatase activity was similar to that employed by Fell and Robison [1930]. The tissue was transferred to a cover-slip and dried for 18 hours over sulphuric acid in an evacuated desiccator. It was then weighed on a micro-balance after attaining constant weight in air and transferred to a tube containing 2 cc. of water and a small drop of chloroform. The cover-slip was then reweighed and the weight (W) of tissue thus obtained. The tissues were kept in the chloroform water at room temperature for 3 days; 1 cc. of 0.2 M solution of sodium glycerophosphate (p_H 8.7) was added together with a small drop of chloroform. The further procedure and calculation of the index (A/W) of phosphatase activity

has been described by Fell and Robison [1929]. The ratio A/W expresses the amount of hydrolysis as mg. P effected by 1 mg. of tissue (dry weight) at a temperature of 37° and p_H 8.6.

RESULTS.

In all cases the transplants survived as proved by histological examination and presented the characteristic histological appearance already described [Huggins, 1931]. In the 16-day specimen "osteoid" tissue or fibroblasts with large, swollen, deeply staining cytoplasm surrounded by eosinophilic oedematous tissue was found near the transplanted epithelium: no bone was found at this time or before. In the 18- and 26-day specimens, a rather large amount of bone was found adjacent to the transplanted epithelium. This had the characteristic features of membrane bone.

In the preliminary control tissues (Table I) phosphatase activity could not be demonstrated in the fibrous tissue of the rectus sheath or the muscular portion of the bladder wall. A definite but small value (about 0.03) for A/W was obtained for the bladder epithelium. Variations between A/W values of 0.01 and 0.03 in the whole bladder wall can probably be explained by variations in the amount of epithelium included, since the muscle does not contain the enzyme.

Table I. *Phosphatase activity of various tissues removed at operation as controls.*

Tissue	W mg.	A mg. P	A/W
Whole thickness of bladder wall	3.39	0.11	0.03
"	19.1	0.05	< 0.01
"	14.2	0.10	< 0.01
Bladder epithelium alone	5.04	0.14	0.03
"	4.5	0.10	0.02
"	3.7	0.09	0.03
"	3.0	0.05	0.02
"	3.6	0.12	0.03
"	3.7	0.55	0.01
"	13.0	0.06	< 0.01
Bladder musculature alone	6.6	0.02	< 0.01
"	9.3	0	0
Connective tissue rectus sheath	10.6	0.01	< 0.01
"	3.5	0	0
"	5.9	0	0
"	8.9	0	0
"	5.7	0	0

Of the tissues of the transplant containing bladder epithelium histologically (Table II *b*) no activity could be demonstrated at 5 or 9 days. In similar specimens at 12, 13 and 16 days small amounts of phosphatase were demonstrated and the A/W value was of the order of 0.01 except in one instance where it was 0.05.

Specimens of connective tissue adjacent to but not including bladder epithelium (Table II *a*) from 12 to 26 days contained even smaller amounts of the enzyme, ranging from 0 (14 times) to a maximum value of 0.05 (once).

Table II.

Phosphatase activity of connective tissue adjacent to bladder epithelium transplant.

Time after operation	W mg.	A mg. P	A/W
(a) No bladder mucosa or bone present.			
12	5.0	0.25	0.05
12	7.0	0.07	0.01
13	19.7	0.25	0.01
13	22.2	0.51	0.02
13	13.2	0.06	<0.01
16	36.0	0.02	<0.01
16	12.0	0.20	0.02
16	42.0	0.005	<0.01
16	14.0	0.05	<0.01
16	9.0	0.13	0.01
18	5.07	6.192	0.04
18	6.0	0.187	0.03
18	2.7	0	0
18	12.0	0	0
18	2.0	0	0
18	4.2	0	0
26	3.6	0	0
26	4.4	0	0
26	2.2	0	0
26	1.4	0.042	0.03
26	7.0	0.033	<0.01
26	5.2	0.033	<0.01
26	4.0	0.055	0.01

Phosphatase activity of bladder epithelium transplanted to connective tissue.

Time after operation	W mg.	A mg. P	A/W
(b) Bladder mucosa but no bone present.			
5	1.8	0	0
5	3.0	0	0
9	3.8	0	0
9	3.3	0	0
9	5.9	0	0
12	10.0	0.113	0.01
12	11.0	0.09	<0.01
12	23.0	0.22	0.01
12	4.5	0.055	0.01
13	14.5	0.78	0.05
16	10.0	0.13	0.01
16	22.0	0.18	<0.01
(c) Bladder mucosa and bone present.			
18	1.0	0.41	0.41
18	0.8	0.099	0.12
18	3.1	6.80	0.22
18	0.8	0.198	0.25
26	1.3	0.20	0.16
26	1.0	0.13	0.13
26	1.5	0.18	0.13
26	2.7	0.57	0.21
26	1.6	0.25	0.16

Contrasting sharply with these index values in unossified tissues and controls, the 18- and 26-day tissues containing bone (Table II c) without exception contained large amounts of phosphatase; the A/W value varied from 0.12 to a maximum of 0.41. These values are of the same order of magnitude as those obtained by Fell and Robison [1929, 1930] for normal and embryonic ossifying cartilage and bone. The variations can be accounted for in part by the different amounts of soft tissues in relation to bone in the material examined. In the presence of bone, therefore, phosphatase was present in amounts 2.4 to 8 times the maximum present in the highest of non-ossified tissues and 10 to 20 times the mean values for these tissues (Table III).

Table III. Summary of ratios of phosphatase to dry weight A/W .

	Controls	Transplantation experiment		
	Bladder mucosa	Rectus sheath	Rectus sheath bladder epithelium. No bone	Rectus sheath bladder epithelium. Bone
Maximum	0.03	0	0.05	0.41
Minimum	<0.01	0	0	0.12
Mean	0.02	0	0.01	0.20

SUMMARY.

The histological and chemical relationships of the enzyme (phosphatase) effecting hydrolysis of certain phosphoric esters were studied in the various stages of heterotopic osteogenesis in transplants of the urinary bladder epithelium to the abdominal fibrous tissue in the dog. In the presence of bone so formed the ratio of phosphatase activity to dry weight was very high, at least 2·5 to 8 times higher than the maximum, and 10 to 20 times the mean values obtained for the control tissues without bone.

The writer is greatly indebted to Dr R. Robison for many kindnesses, suggestions and criticism. He also wishes to thank the Lister Institute by whom the expenses of this investigation were defrayed and Prof. C. L. Evans for the use of the facilities of the Department of Physiology, University College, London, where the surgical work was done.

REFERENCES.

- Fell and Robison (1929). *Biochem. J.* **23**, 767.
— — (1930). *Biochem. J.* **24**, 1905.
Huggins (1930, 1). *Proc. Soc. Exp. Biol. Med.* **27**, 349.
— (1930, 2). *Proc. Soc. Exp. Biol. Med.* **28**, 125.
— (1931). *Arch. Surg.* **22**, 577.
Martland and Robison (1924). *Biochem. J.* **18**, 765.
Robison (1923). *Biochem. J.* **17**, 286.
— Macleod and Rosenheim (1930). *Biochem. J.* **24**, 1927.
— and Soames (1924). *Biochem. J.* **18**, 740.
— — (1930). *Biochem. J.* **24**, 1922.
Roseberry, Hastings, and Morse (1931). *J. Biol. Chem.* **90**, 395.

LXXXI. FAT METABOLISM IN MUSCULAR EXERCISE.

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A RISE in the blood-fat during muscular exercise was first described by Patterson [1927], whose experiments left no doubt as to the reality of the phenomenon. His suggestion that the rise in blood-fat is a response to the demand of the working muscles for fat agrees with the observation of Lafon [1913] that the blood leaving a working muscle contains less fat than the blood entering it. The phenomenon of an alteration in the lipoid content of the blood during muscular exercise appears to be of importance not only from the point of view of blood chemistry, but also on account of the controversy which has arisen as to whether muscle is capable of utilising fat as a source of energy. The experiments described in this paper have been undertaken to extend the work of Patterson by elucidating the source and the nature of the extra fat which appears in the blood, the cause of its appearance, the chemistry of muscle with respect to the use of fat, and the relationship between the rate of utilisation of fat and its concentration in the blood.

EXPERIMENTAL.

Preparation of the subject. The subjects, with one or two exceptions noted later, were healthy young men, used to taking moderate exercise, and were on a normal mixed diet. At the time of the experiment they had fasted for 13–15 hours. They had either spent the night in the laboratory, or had rested there in bed since early morning. In either case, a state as nearly basal as possible was assured at the commencement of the experiment. For those experiments involving measurements of the gaseous exchange, this condition was obviously essential, since it provided the only possible base line from which to measure the excess metabolism during the exercise. Further, preliminary experiments showed that any previous activity on the part of the subject considerably modified the effect of a given amount of muscular work on the level of the blood-fat. These precautions were not observed by Patterson in all of his experiments, which doubtless explains some of the quantitative discrepancies between his results and those about to be described.

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Mode of exercise. The subjects were exercised on a bicycle ergometer at a steady rate. To most of the subjects cycling was an unaccustomed form of exercise, and it was found impossible for them to work for long at a greater rate than about 1100 kg. m. per minute. Short periods of work such as were employed by Patterson in his running experiments were not feasible, since amount rather than rate of work seemed to be the important factor. To avoid a training effect the same subject was not used for more than one experiment except after the lapse of a considerable period.

Experiments involving measurement of respiratory exchange. After the subject had reached basal conditions, and had emptied his bladder, his expired air was collected in a 200 litre spirometer for a period of 25 minutes. A sample of blood was drawn and the bladder was again emptied. The work was then begun, and at intervals during its progress the expired air was collected for a period of 1 minute, the subject being connected to the spirometer for this purpose without interruption of the steady state of work. A number of preliminary experiments satisfied us that extrapolation from the results of these separate collections gave nearly as accurate a figure for the gaseous exchange over the whole period as the much more cumbersome and uncomfortable procedure of collecting all the expired air throughout the exercise. The oxygen consumption reached a steady value about 5 minutes from the commencement of exercise, and it was therefore necessary only to confirm this in each case by taking two samples during the latter part of the exercise with, in addition, one sample at the second or third minute of exercise. Special care was needed at the end of the exercise on account of the sudden alteration in the respiratory exchange at the beginning of recovery. On the completion of the work, the subject immediately lay down and was once more connected to the spirometer. A second sample of blood was then taken. Collection of the expired air was continued until the subject had again reached a basal condition, as shown by pulse and respiration rate, and minute volume of expired air. At the end of this period—usually an hour was allowed—the subject again emptied his bladder and a third sample of blood was procured.

Experiments not involving measurement of respiratory exchange. In two series of experiments it was not feasible to measure the respiratory exchange. The first of these was undertaken in order to determine the effect of a short period of work, following immediately after apparent recovery from one of sufficient magnitude to produce a rise in the blood-fat. In one or two of these the respiratory exchange was determined over the first part of the experiment, but it was found that the discomfort of the mask, worn over such a long period of time, precluded the proper return of the subject to basal conditions after the second period of exercise. In these experiments blood was drawn before and after each period of exercise. The second series had as its object the determination of changes in the blood during the progress of exercise, and blood samples were withdrawn, not only before and after, but at intervals during the performance of work. This procedure necessitated

interruption of the exercise, and, though the withdrawal of samples was performed as expeditiously as possible, the interruption rendered fallacious any measurement of the respiratory exchange with the means at our disposal.

Chemical methods. The respiratory exchange was calculated in the usual way by analysis of samples removed from the spirometer.

The urine was analysed for total nitrogen and urea-nitrogen in order to determine whether any of the extra metabolism during exercise and recovery was due to protein breakdown. It may be said at once that no increase in the rate of nitrogen excretion was found during the experiments (Table I).

Table I.

Subject	Nitrogen excreted during exercise and recovery	Nitrogen excreted during same time under basal conditions
	g.	g.
R.G.	1.23	1.36
D.M.D.	0.80	0.80
C.P.S.	0.79	1.03
J.B.R.	1.28	1.05
D.S.	1.29	1.25
R.E.I.	1.08	1.18
De V.	0.75	0.84
W.McL.	0.35	0.49

Usually, in fact, the urine secreted during the period of exercise and recovery contained rather less nitrogen than that for the corresponding basal period. This slight decrease, no doubt, is to be attributed to an increased elimination of nitrogen by the skin.

In the various blood samples, total fat, soap, total cholesterol, lipid phosphorus, the iodine number of the fatty acids, and the carbon dioxide-combining power were estimated.

The method used to estimate the total fat in blood was a modification of that of Stewart and White [1925]. This method has been criticised by Stoddart and Drury [1929]. In the work reported here no difficulty was found in obtaining duplicate analyses agreeing to within 5% by using the technique of Stewart and White, and several other workers in this laboratory have also used this method with good results. It has long been realised, however, that this method of analysis estimates the phosphoric acid from the phospholipins as well as the fatty acids obtained from the fats and phospholipins by hydrolysis. To overcome this difficulty, a modification of the original technique was devised, involving filtration---the same device as was introduced by Stoddart and Drury [1929]. As before, the total lipoids were extracted from blood with alcohol-ether mixture, and were saponified by evaporation almost to dryness with 5 cc. of *N*/10 sodium hydroxide. Whereas in the original method, this alkali was exactly neutralised by adding 5 cc. of *N*/10 hydrochloric acid, a slight excess of acid was now added, with thorough shaking to aid liberation of the free fatty acids; the mixture was warmed to produce coagulation of the fine particles, and filtered through a fat-free filter-paper. The precipitated fatty acids were washed three times with 1 cc. of 5% sodium chloride. The precipitate was then dissolved in alcohol and the solution was made up to 10 cc. 1 cc. of this solution was titrated with *N*/10 sodium hydroxide, using the Rehberg burette. From the mean of three titrations, the blood-fat was calculated as tripalmitin.

The use of this modification gave results which were consistently lower than those given by the original Stewart and White method. If the phosphoric acid of the phospholipins of the blood were titrating as a monobasic acid, the difference in the results given by the two methods would

be accounted for. Table II shows the results of analyses of bloods in which fat was estimated by both methods, and the lipid phosphorus was also determined. It shows how closely the difference in the fat content as determined by the two methods agrees with the fatty acid which would correspond to the lipid phosphorus if it were titrating as a monobasic acid.

Table II.

Blood sample	Fat (mg./100 cc.)		Lipid phosphorus (mg./100 cc.)	
	Original method	Modified method	Observed	Calculated
1	654, 657	501, 521	15.0	16.0
2	659, 679	444, 454	20.1	24.0
3	426, 429	331, 338	12.3	10.4
4	461, 491	320, 350	16.4	16.0
5	659, 660	446, 442	22.5	24.0

The method of Stewart and White [1929] was used for the estimation of soaps, that of Myers and Wardell [1918] for cholesterol, that of McClure and Huntsinger [1928] for iodine values, that of Van Slyke and Neill [1924] for carbon dioxide-combining power, and that of Briggs [1924] for lipid phosphorus.

RESULTS AND DISCUSSION.

The utilisation of fat by muscle. The use of the R.Q. for determining the nature of the fuel used during muscular exercise demands as a primary condition that the body shall be in exactly the same condition at the beginning and end of the measured period except for the loss of fuel. The probability is that this condition, except perhaps in very mild exercise of short duration, is never entirely fulfilled. Not only must the oxygen consumption and the R.Q. have returned to their original levels—which involves the inclusion of a recovery period—but so must the lactic acid, carbon dioxide content and combining power of the blood. Even so, the conditions before and after exercise may not be strictly comparable, since even moderate exercise involves the excretion of lactic acid, and it is not known how this excretion affects the urinary content of bicarbonate ions or the ammonia/urea ratio. Such factors as these, however, though they may introduce considerable errors in experiments of short duration where the gas volumes dealt with are small, cannot have a very great effect on the longer experiments reported here. The particular ones mentioned above, in fact, would tend to make the observed R.Q. higher than the actual one. Hence though they, and others of the same kind, may afford at any rate a partial explanation of the very high respiratory quotients observed by Best, Furasawa and Ridout [1928], in violent exercise of very short duration, they do not invalidate the deductions drawn here.

In Fig. 1 the amount of work performed by a number of subjects is plotted against the R.Q. for exercise and recovery at the end of which the oxygen consumption, R.Q., and carbon dioxide-combining power of the blood had returned to normal. The figures used in constructing this curve were, of course, those for the extra metabolism due to the exercise, the basal metabolism having been deducted from the gross figures.

In three cases the R.Q. for small amounts of work was very much lower

than was expected, a result which indicates, if the conclusions drawn later are justified, a much earlier usage of fat. In these cases there appears to be either a relative inability to utilise glycogen in the normal way or a very low store of glycogen, and it is significant that all three are cases of diabetes mellitus. Since the evidence in the literature [Lusk, 1928] indicates that in

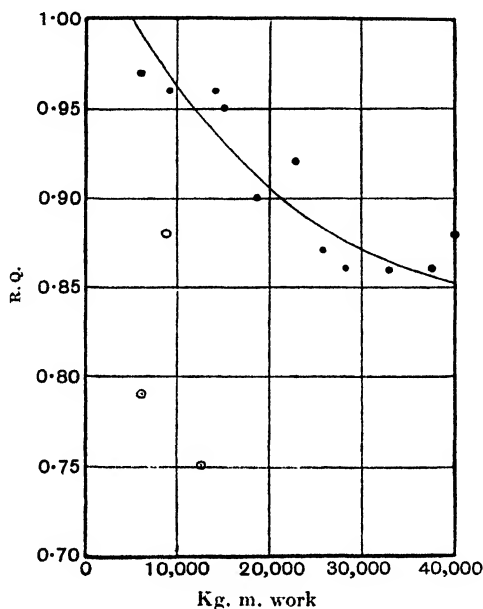


Fig. 1. Relationship between the amount of work and the R.Q. for exercise and recovery.

• Normal.

○ Diabetic.

this condition the muscle-glycogen is maintained at the usual level long after the remaining stores are depleted, and since the cases reported here were under control, it is reasonable to conclude that the fault lay in the utilisation of carbohydrate rather than in its lack. It is significant that glycosuria was not produced by the exercise.

With small amounts of work (4000–5000 kg. m.), the excess R.Q. was at or very near 1.0 (in two cases values appreciably above this were obtained), but with increasing amounts of work, the R.Q. for exercise and recovery steadily fell. In spite of the fact that twelve individuals are represented in the curve, the proportionality between the amount of work performed and the R.Q. is definitely marked, and goes far to support the substantial truth of the figures. It is perhaps advisable to reiterate here that the rate at which the different subjects performed the work was nearly constant, the tension on the ergometer bicycle and the rate of pedalling being kept as nearly constant as possible. The conclusion seems justified that, although the muscles prefer carbohydrate as a fuel, they are fairly rapidly forced to fall back on some other

fuel of lower R.Q. In no case did the results of urine analysis indicate any usage of protein.

There remains fat, which, therefore, one is forced to suppose is used by the muscles as a secondary source of energy. The alternative hypothesis that extra fat is used in the basal processes, thereby liberating carbohydrate for use in the working muscles, is disproved by a consideration of the amounts involved. Thus in one experiment, with R.Q. 0.96 for exercise and recovery, the relevant data are as follows. This experiment has been chosen for illustrative purposes since, with the small amount of fat utilisation involved, it offers a less convincing example than those experiments with lower R.Q.

R.Q. for exercise and recovery	0.96
Oxygen consumption for exercise and recovery	24.17 litres
Duration of exercise and recovery	85 minutes
Calculated fat used during exercise and recovery	3.31 g.
Heat equivalent of fat used	31.35 cal.
Basal oxygen consumption for 85 minutes	26.28 litres
Basal R.Q.	0.77
Nitrogen excretion during 85 minutes	1.097 g.
Carbohydrate used in basal metabolism during 85 minutes	3.55 g.
Heat equivalent of carbohydrate	14.86 cal.

The amounts of fat and carbohydrate are calculated from the tables given by Lusk [1928]. It is evident that the energy available from the whole of the carbohydrate used for basal metabolism during the period of exercise and recovery would not, if entirely diverted to the working muscles, obviate the necessity of their calling upon fat.

The R.Q., though it allows the deduction that fat is used as a fuel by the working muscles, gives no information as to whether the fat is used directly or only by conversion to carbohydrate.

The controversy as to which of these alternative routes is followed by the fat used in muscular exercise hinges largely on the problem of the relative efficiency of fat and carbohydrate.

It is rightly argued that if fat is utilised by direct oxidation, muscular efficiency should be unaltered when it replaces carbohydrate; on the other hand, utilisation only of carbohydrate derived from fat necessarily implies a loss of energy, and therefore a decreased efficiency. Moreover, the loss of efficiency should be the same for every individual. In considering the magnitude of such a hypothetical loss of efficiency, one is faced with the difficulty that the conversion of fat to carbohydrate in the animal body has never been conclusively proved, and that those workers whose experiments have seemed to demonstrate the possibility of such a conversion have thrown no light on the mechanism. The smallest wastage of energy, as has often been pointed out, would occur in the unlikely event of conversion of the whole of the carbon of the fat molecule into carbohydrate, and even then some 25 % of the total available energy would be lost.

The classical work of Krogh and Lindhard [1920], in which the maximum

loss of efficiency was found to be only 11 %, is often cited as disproving the fat to carbohydrate conversion hypothesis. The original authors, however, are more cautious, and merely state that their experiments cannot be used as proof that fat is necessarily converted to carbohydrate before being utilised by working muscle.

The change over from carbohydrate to fat as muscular fuel is, of course, not sudden and complete. Even when fat has become the preponderating source of energy, carbohydrate is still being used though in diminishing amount. This is shown in Fig. 2, in which the amounts of fat and carbohydrate used for various amounts of work (and recovery) are plotted against the amounts of external work performed. The figures for the amounts of fat and carbohydrate used are calculated in the usual way from the observed excess oxygen consumption and R.Q. It is evident from the curves that as the amount of work increases the amount of fat used increases, but so also does the amount of carbohydrate. Even with R.Q. 0.80, carbohydrate is still being used; the R.Q. is not simply the mean result of the early exclusive use of carbohydrate and the later exclusive use of fat.

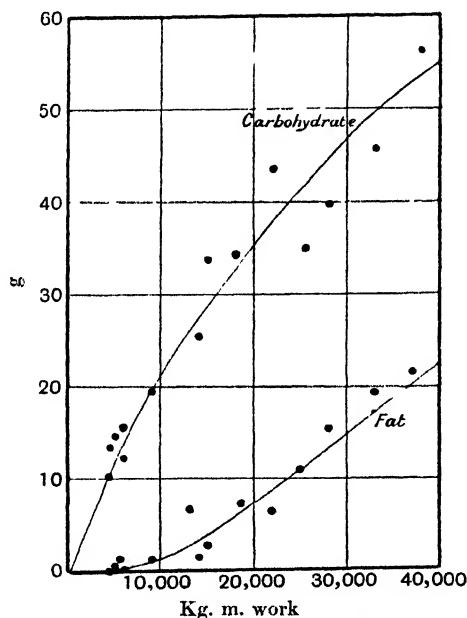


Fig. 2. Relationship between the amount of external work and the amounts of fat and carbohydrate oxidised.

The R.Q. for a complete period of work and recovery can never be that of fat alone—except possibly in the case of a complete diabetic, and then only on the assumption that the diabetic cannot oxidise carbohydrate or lactic acid. Hence the loss of efficiency to be expected is always less, and, with R.Q. 0.80

and over, very much less than the calculated 25 %. Thus, in one of the experiments made by the present authors, the R.Q. and oxygen consumption for work and recovery showed the utilisation of 56.8 g. of carbohydrate and 21.56 g. of fat, with R.Q. 0.86. A simple calculation shows the expected loss of efficiency to be only 11.5 %.

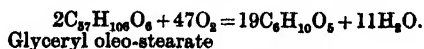
In the experiments of the present authors no loss of efficiency was observed when work was performed at the expense of fat (Table III). It is admitted that the experiments were not specially designed for the purpose of showing such change in efficiency, and that in no case was a series of experiments undertaken upon the same individual with varying amounts of work and at varying R.Q. Nevertheless, since the conversion of fat into carbohydrate involves a definite wastage of energy irrespective of individuals, it seems fair to suppose that any such wastage would be shown in the average results from a large number of subjects. Since the subjects were not trained cyclists, the fact that only one experiment was performed upon each was an actual advantage, for it eliminated any fallacy due to training. The numbers reported here are not, it is true, large, but, as they show no suggestion of a wastage of energy with the lower R.Q., and as the distribution in each series is nearly the same, it has been thought worth while to report them (Table III).

Table III.

R.Q.	1.0	0.95-0.99	0.90-0.94	0.85-0.89	0.80-0.84
Lowest			4.76	4.23	4.32	4.11	4.06
Highest			5.71	5.15	5.38	5.65	5.70
Mean (no. of expts)			5.12 (6)	4.82 (4)	4.86 (6)	4.78 (7)	4.60 (6)

They differ radically, of course, from those of Krogh and Lindhard [1920], and it seems possible that the difference may lie in the fact that all the subjects dealt with here were on an ordinary mixed diet, while Krogh and Lindhard used high and low carbohydrate diets to obtain the variations in R.Q. Moreover, the rate of work in the experiments reported here was much greater than in those of Krogh and Lindhard.

A wastage of energy in the utilisation of fat should become apparent not only in the calculated efficiency, but also in another way. The wastage, if fat is converted to carbohydrate, is due to some such process as loss of hydrogen which is oxidised to water. Thus, in the most favourable conversion,



The utilisation by the muscles of 1 g. of glycogen derived from fat demands, according to this equation, the consumption of 1.099 litres of oxygen instead of the 0.828 litre required by the glycogen itself—an oxygen wastage of 31.5 %. On the other hand, the direct utilisation of fat involves no loss of oxygen, and the curve showing oxygen consumption per calorie equivalent of work done is simply that of the “calorific value” of oxygen calculated by Zuntz and Schumburg [1901]. These two curves for the consumption of oxygen

per calorie equivalent of work are shown in Fig. 3. In these curves allowance is made for the simultaneous use of carbohydrate, the relative amounts of the two fuels being calculated on the basis of Fig. 1. The experimentally determined values are also shown in Fig. 3, and, though there is a certain amount of scattering due to the employment of many different individuals, it is evident that by far the closer approximation is given by the curve corresponding to direct utilisation of fat.

In the curve, as in the table of efficiencies, are included the results from the three diabetic subjects, and it is interesting to note that in spite of their

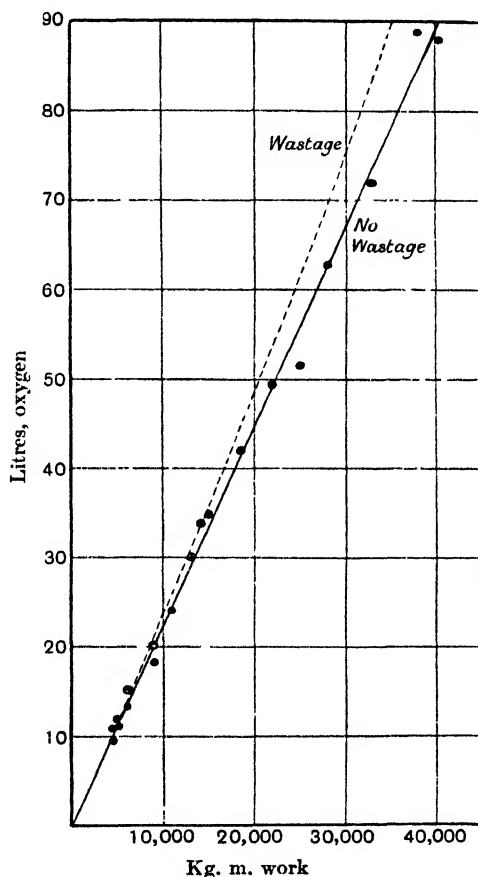


Fig. 3. Relationship between the amount of work and the oxygen consumption for work and recovery. The continuous line represents the volume of oxygen expected if there is no wastage consequent upon the utilisation of fat. The dotted line represents the oxygen consumption calculated on the basis of a 30 % wastage for the conversion of fat to carbohydrate.

● Normal.

⊙ Diabetic.

abnormally low R.Q., indicating the utilisation of an abnormally high proportion of fat, they show no deviation from the normal efficiency.

Though no finality is, of course, claimed for these results, they tend very

definitely to oppose the view of the necessary conversion of fat to carbohydrate prior to its utilisation by the working muscles, and to support the view that fat and glycogen are alternative fuels although the latter appears to be used preferentially.

The theoretical need for a conversion of fat to carbohydrate has, indeed, been greatly weakened by the recent work of Eggleton and Eggleton [1927] on creatinephosphoric acid and of Lundsgaard [1930] on muscle poisoned with iodoacetic acid, which appears to show that the breakdown of glycogen to lactic acid can no longer be regarded as an essential in muscular contraction. Even if the glycogen-lactic acid cycle be considered essential, it may well be that fat, by its direct oxidation, is capable of sparing lactic acid, leaving more for resynthesis to glycogen and so conserving the supplies of carbohydrate.

It is perhaps worth pointing out, though with diffidence in view of modern ideas as to the function of insulin, that if it be admitted that diabetes mellitus involves a failure to oxidise carbohydrate, calculations from cases of nearly complete diabetes show that such diabetics do not convert fat to carbohydrate. Such a conclusion follows, *e.g.* from the data in the case reported by Geyelin and Du Bois [1916], in which the observed respiratory exchange and heat production can only be explained in this way. The diabetic can oxidise fat as far as acetoacetic acid, and it is therefore a fair conclusion that any conversion of fat to carbohydrate is possible only after this stage, *e.g.* by oxidation to acetaldehyde followed by aldol condensation. Such a conversion would involve a wastage, not of 25 %, but of 80 % of the energy of fat, and would render quite impossible of accomplishment the amounts of work actually observed.

The variation in blood-fat in muscular exercise. The results of blood analysis (Table IV) show that the majority of normal subjects do experience some increase in the blood-fat, when, under fasting conditions, they perform work in excess of approximately 8000 to 10,000 kg. m. This figure is only approximate, since the exact point at which the rise in the blood-fat occurs depends to a considerable extent on the rate at which the work is performed. Table IV gives typical results of blood analyses. The experiments on normal subjects (*A*) show that the rise in blood-fat does not occur in every case (*e.g.* C.P.S., D.S.); when it does occur, it is transient, and has disappeared at the end of the recovery period.

Patterson [1926] observed that diabetic subjects almost invariably showed a fall in the blood-fat, even with the relatively small amounts of work they were able to perform. All his diabetic subjects, however, had initially an abnormally high blood-fat, a state of affairs which is usually present in this disease. The experiments summarised in Table IV *B* show that this fall in the blood-fat produced by exercise is characteristic, not of diabetes mellitus, but of the incidental high blood-fat level. It occurred in subjects whose fasting blood-fat was abnormally high, but who were apparently healthy in all other respects. Moreover, in two diabetic cases (Table IV *C*), with the basal blood-

Table IV.

	Subject	Work kg. m.	Blood sample	Fat mg./100 cc.	Cholesterol mg./100 cc.	Lipoid P mg./100 cc.	Soap mg./100 cc.	
<i>B</i>	R.G.	5,500	<i>a</i>	492	134	—	40.0	
			<i>b</i>	433	139	—	37.9	
			<i>c</i>	481	128	—	41.2	
			<i>d</i>	579	133	—	41.2	
	C.P.S.	14,966	<i>a</i>	689	136	21.7	27.9	
			<i>b</i>	693	136	21.9	17.6	
			<i>c</i>	522	137	21.6	24.6	
			<i>d</i>	562	137	20.0	27.2	
	D.S.	18,564	<i>a</i>	478	143	20.7	—	
			<i>b</i>	453	144	22.3	—	
			<i>c</i>	450	125	23.1	—	
			<i>d</i>	481	127	20.3	—	
	R.E.I.	19,123	<i>a</i>	607	118	13.3	30.7	
			<i>b</i>	791	114	12.1	30.2	
			<i>d</i>	583	118	11.6	31.2	
	R.G.	28,050	<i>a</i>	480	145	—	30.7	
			<i>b</i>	588	144	—	45.7	
			<i>d</i>	434	146	—	39.8	
		R.E.I.	33,000	<i>a</i>	511	118	18.8	23.9
			<i>b</i>	674	118	16.5	25.2	
		<i>c</i>	467	—	19.0	25.0		
		<i>d</i>	425	—	15.0	23.9		
	De V.	37,150	<i>a</i>	376	120	15.3	23.6	
		<i>b</i>	511	142	15.2	24.6		
		<i>c</i>	464	127	17.2	24.1		
		<i>d</i>	460	122	17.5	24.5		
	D.M.D.	4,300	<i>a</i>	853	208	21.7	28.2	
			<i>b</i>	685	209	21.9	26.7	
			<i>c</i>	522	208	21.8	22.6	
			<i>d</i>	562	207	21.7	27.2	
	J.B.R.	14,000	<i>a</i>	1103	139	15.5	29.7	
			<i>b</i>	831	141	18.3	35.7	
			<i>c</i>	694	140	15.1	42.7	
			<i>d</i>	899	139	14.5	42.7	
<i>C</i>	J.McC.	8,600	<i>a</i>	770	—	—	—	
			<i>b</i>	825	—	—	—	
	W.McL.	12,300	<i>a</i>	690	—	—	—	
			<i>b</i>	812	—	—	—	
<i>D</i>	F.P.C.	22,250	<i>a</i>	310	119	15.9	36.0	
			<i>b</i>	660	121	15.5	34.7	
			<i>c</i>	510	121	—	35.7	
			<i>d</i>	410	120	15.6	35.2	

a = Before work.
b = After work.

c = After partial recovery.
d = After complete recovery.

fat not much above the normal level, exercise produced a small but definite increase as easily as in normal subjects. Nor, probably, is it mere coincidence that in one case the converse appeared to hold, and that a healthy subject with an abnormally low fasting blood-fat level showed an abnormally great increase after a moderate amount of work (Table IV *D*). These results lead to the conclusion that the blood-fat content tends to reach the normal level before it shows the increase usually given by healthy individuals. This preliminary change takes place under the influence of moderate work, definitely

below the amount required to produce a change in the blood-fat content of subjects whose basal blood-fat is within the normal range.

Those experiments in which the subject performed a second period of work after recovery from the first (Table V) demonstrate that a rise in the blood-fat is rather more easily obtained during the second period than during the first. This point is of importance as emphasising the necessity of obtaining basal conditions before starting the exercise if comparable results are to be obtained from a series of experiments on different individuals. It also tends to support the contention that the increase in blood-fat during exercise is in some way connected with the utilisation of fat by the muscles. There can be no doubt that at the commencement of the second period of exercise the body stores of glycogen have been somewhat depleted, and hence the muscles—if they can use fat—must call for fat earlier, though only slightly so, than in the first period.

Table V.

Subject	Blood sample	Fat mg./100 cc.	Cholesterol mg./100 cc.	Lipoid phosphorus mg./100 cc.
R.G.	Basal	611	145	14.6
	After 1st work (28,000 kg. m.)	719	144	14.2
	After recovery	565	146	14.6
	After 2nd work (11,000 kg. m.)	619	147	14.3
R.E.I.	Basal	607	118	13.3
	After 1st work (19,000 kg. m.)	791	114	13.1
	After recovery	583	118	11.6
	After 2nd work (9000 kg. m.)	647	118	13.7

The results so far described confirm the statement that muscular exercise is accompanied by an increase in the blood-fat, though they show that this is not an invariable result. It was desired, however, to find in what constituents of the total blood-fat the increase occurred. The analyses (Tables IV and VI) showed that the increase lay solely in the triglyceride fraction, which, according to Channon and Collinson [1929], forms a very small part of the total blood-lipoids under resting conditions. While the total fatty acids increased, the lipid phosphorus and the cholesterol showed either no change or only small and inconstant variations. The free fatty acid (soap) content of the blood remained unaltered, and though there was possibly an increase in the amount of esterified cholesterol, any such increase was quite incapable of accounting for the observed increase in the total fatty acid. Figures in support

Table VI.

Subject	Total cholesterol mg./100 cc.	Fat required to esterify completely mg./100 cc.	Rise in fat mg./100 cc.
R.E.I.	118	13.7	163
FF.P.C.	121	14.0	350
De V.	142	16.5	135
R.G.	144	16.7	108
R.E.I.	114	13.2	184
R.M.L.	136	15.8	92
R.G.	129	15.0	220

of this statement are given in Table VI, which is based on the ratio of esterified to unesterified cholesterol given by Channon and Collinson [1929].

Finally, since Patterson's figures were obtained by the use of the original Stewart and White method, it is important to note that exactly similar results are obtained—as in the experiments here described—by use of the modified method which estimates only fatty acids and does not include phosphoric acid. Otherwise the apparent increase in the blood-fat during exercise might conceivably have been regarded as due merely to an altered state of dissociation of the phospholipins.

The iodine value of the blood-fat before and after exercise was determined in a number of cases (Table VII), and although it was found to be very variable for different subjects, it altered only very little in the same subject as a result of exercise, and in no case did it show a rise. This suggests very strongly that the source of the fat poured into the blood stream in response to stimulation by exercise is the adipose tissue and not the liver.

Table VII.

Subject	Work	Fat mg./100 cc.	Iodine value	Subject	Work	Fat mg./100 cc.	Iodine value
T.F.	Basal	426	63	E.B.H.	Basal	364	26
	11,550 kg. m.	421	65		13,250 kg. m.	374	24
	16,885	661	52		20,500	485	14
	24,650	448	32		26,650	387	26
E.G.	Basal	660	84	A.T.B.	Basal	444	22
	12,750 kg. m.	690	65		10,250 kg. m.	454	27
	18,965	865	62		16,250	454	34
	28,000	757	78		23,050	444	36

The variation of the blood-fat during muscular exercise. Those experiments in which blood-fat estimations were made during the performance of work showed a curious result which ultimately shed light on the marked individual variations and apparently haphazard responses obtained in previous experiments. It appeared that, instead of the blood-fat steadily increasing during exercise, it first rose and then fell. This result was obtained in a considerable number of individuals, and typical cases are shown in Fig. 4. When the results were plotted together as a composite curve, it appeared that actually there was a rise in the blood-fat, followed by a fall and then by a second rise (Fig. 5, dotted line). Moreover, the results obtained from nearly all the previous experiments could be fitted to this curve. Certain exceptions were noted, as when the rate of work differed markedly from that usually employed, or when the subject had an abnormal resting blood-fat.

The shape of the composite curve had been disguised in the later individual experiments shown in Fig. 4 by the fact that only three points were obtained. To test whether the exceptions to this composite curve were really due to the one observed variation in experimental technique—the rate of work—a second series was undertaken in which the subjects worked at a rate of 1100 kg. m. per minute instead of the 900 kg. m. per minute which had

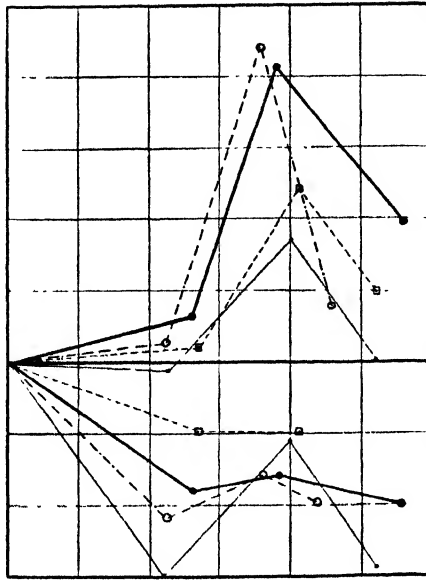


Fig. 4. The blood-fat and carbon dioxide-combining power during exercise. Individual cases. (Ordinates as for Fig. 5.)

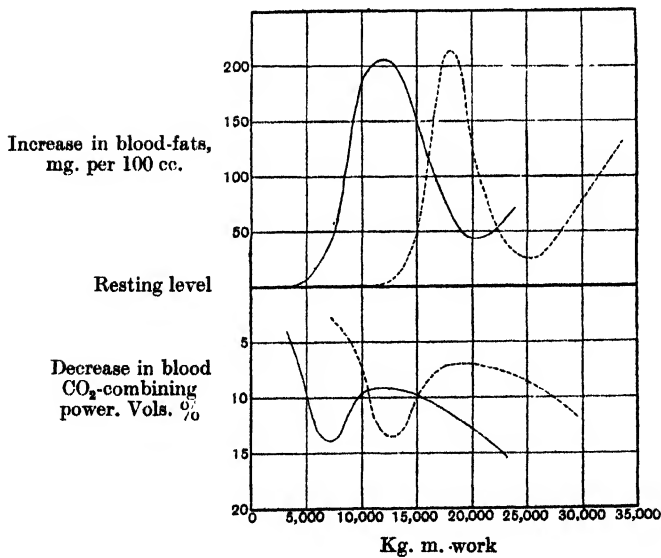


Fig. 5. Composite curves showing changes in the blood-fat concentration and the blood carbon dioxide-combining power during exercise. The dotted line represents changes at 900 kg. m. per minute, the continuous line at 1100 kg. m. per minute.

previously been the rule. The results of blood-fat determination from this series fell on a second composite curve (the continuous line in Fig. 5) of exactly the same shape as the first, but showing the changes in blood-fat concentration to take place after a smaller amount of work than previously. These variations in the blood-fat during work were entirely unexpected, and could not be correlated with a steadily increasing consumption of fat by the working muscles. Yet experiment had shown that such a steadily increasing consumption did indeed exist (Fig. 2).

It became necessary, therefore, to discard the simple suggestion that the increase in blood-fat during exercise was a direct response to a demand by the muscles for an alternative fuel consequent upon the utilisation of glycogen. Attention was thus directed to the discovery of some stimulus which could act as a connecting link between the two phenomena. The production of lactic acid had suggested itself as a possible causative factor in the rise in blood-fat, which, at that time, was all that had been observed. For ease of measurement the carbon dioxide-combining power of the blood had been determined in all samples. The results of these analyses showed at first somewhat irregular decreases in the carbon dioxide-combining power of the blood following exercise, but it was only when blood samples were analysed during the course of exercise that any real relationship between the blood-fat and the carbon dioxide-combining power was observed. It then transpired that the carbon dioxide-combining power, instead of falling steadily with increasing amounts of work to the acidosis level, followed a course which was roughly the reciprocal of that followed by the blood-fat (see Fig. 4). A composite curve of all the results obtained, just as in the case of the blood-fats, was sinuous, showing a fall followed by a partial recovery and only then a steady progression to the acidosis level.

The second series of experiments in which blood samples were drawn during the progress of exercise showed a composite curve of exactly the same character as the first. Again, however, as in the case of the blood-fat concentration, the fall occurred at an earlier period of the work. Moreover, in both series, the fall in the carbon dioxide-combining power preceded the rise in blood-fat, and its partial recovery preceded the fall in blood-fat. Whether this time relation between the curve of carbon dioxide-combining power and that of the blood-fat concentration has any real significance, it is too early to say definitely. It is none the less interesting to speculate whether it indicates a causative relationship between the two phenomena.

SUMMARY.

1. When normal healthy men performed muscular work on an ergometer bicycle at rates varying from about 800 to 1200 kg. m. per minute, the blood-fat usually rose after about 8000 kg. m. of work had been done.
2. The increase in the blood-fat concentration appeared earlier with greater rates of work.

3. After recovery from a first period of work, a second period produced a rise in the blood-fat more easily than usual.

4. A high fasting blood-fat in normal or diabetic subjects led to a preliminary fall when work was performed; an abnormally low blood-fat was increased by relatively small amounts of work.

5. This alteration in the blood-fat was confined to the glyceride fraction, and the extra fat was probably derived from the adipose tissue.

6. Continuance of work led to a return of the blood-fat towards normal (at about 18,000 to 21,000 kg. m.), and later to a second rise.

7. The carbon dioxide-combining power of the blood during muscular work of this type followed a course which was roughly the reciprocal of the blood-fat, but the changes in the carbon dioxide-combining power preceded those in the blood-fat.

8. The R.Q. for exercise and recovery was unity for amounts of work up to about 5000 kg. m., and thereafter fell steadily with increasing amounts of work.

9. Analysis of the R.Q., oxygen consumption, and nitrogen excretion showed that protein was not used for work, that carbohydrate continued to be used throughout, though in decreasing amounts, and that fat was used in increasing amounts.

10. There was no relationship between the utilisation of fat and the changes in the blood-fat concentration.

11. No diminution in efficiency was observed at the lower R.Q., and the figures did not suggest any conversion of fat to carbohydrate.

12. Diabetic subjects, though performing work at a lower R.Q. than normals, showed no less efficiency, and no wastage of oxygen.

13. A modification of the Stewart and White [1925] method for the estimation of blood-fat is described.

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REFERENCES.

- Best, Furasawa and Ridout (1928). *Proc. Roy. Soc. Lond. B* **104**, 119.
Briggs (1924). *J. Biol. Chem.* **59**, 225.
Channon and Collinson (1929). *Biochem. J.* **23**, 1212.
Eggleton and Eggleton (1927). *Biochem. J.* **21**, 190.
Geyelin and Du Bois (1916). *J. Amer. Med. Assoc.* **66**, 1532.
Krogh and Lindhard (1920). *Biochem. J.* **19**, 290.
Lafon (1913). *Compt. Rend. Acad. Sci.* **156**, 1248.
Lusk (1928). *Science of Nutrition*, 4th ed. (Saunders, London.)
Lundsgaard (1930). *Biochem. Z.* **217**, 162.
McLure and Huntsinger (1928). *J. Biol. Chem.* **76**, 1.
Myers and Wardell (1918). *J. Biol. Chem.* **36**, 147.
Patterson (1926). M.D. Thesis, Edinburgh.
— (1927). *Biochem. J.* **21**, 985.
Stewart and White (1925). *Biochem. J.* **19**, 840.
— (1929). *Biochem. J.* **23**, 1263.
Stoddart and Drury (1929). *J. Biol. Chem.* **84**, 741.
Van Slyke and Neill (1924). *J. Biol. Chem.* **61**, 523.
Zuntz and Schumburg (1901). *Studien zu einer Physiologie des Marsches* (Berlin), 361.

LXXXII. THE NATURE OF TYROSINASE.

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(Received March 30th, 1931.)

THE view that tyrosinase is a mixture of a number of components has been held by several investigators. Haehn [1920] reported that potato tyrosinase lost its activity on dialysis or ultrafiltration, but regained it on addition of the ultrafiltrate or boiled juice. He concluded that the activator was inorganic in nature. Raper and Wormall [1923], while partially confirming Haehn's finding, noticed that the boiled juice of new but not of old potatoes had an accelerating effect. They also adduced evidence to show that the activator in potato juice is organic in nature.

In an earlier paper [Narayanamurti and Ramaswami, 1929] it was shown that on ultrafiltration of *Dolichos* tyrosinase the residual liquid on the ultrafilter was active and that the addition of the ultrafiltrates to the residual liquid did not cause any acceleration. The following additional results have so far been obtained.

EXPERIMENTAL.

In this investigation the enzyme prepared by three different methods, *viz.* (a) ordinary dialysis, (b) ordinary dialysis followed by ultrafiltration, and (c) ordinary dialysis followed by precipitation with alcohol, has been studied.

Preparation of dialysed extracts. About 200 g. of the finely ground meal of the ungerminated seed of *Dolichos lab lab* were extracted with 600 cc. of distilled water in glass-stoppered bottles at room temperature for 3 days in presence of toluene. After filtering through paper pulp the extract (containing a large quantity of protein) was dialysed in large parchment filters against flowing distilled water for 1 week, when all the protein was precipitated. The solution was then centrifuged to remove suspended matter, the resulting liquid being clear.

Purification by repeated precipitation with alcohol. Dialysed extracts were precipitated with 95 % alcohol, the flocculent precipitate being allowed to settle in an ice-chest and separated by decantation and filtration. The residue on the filter was ground up with distilled water and filtered, alcohol again being added to the filtrate. The whole of the first precipitate does not dissolve and the undissolved residue possesses some activity. The precipitated enzyme is very small in quantity and is dried *in vacuo*.

Ultrafiltration. The ultrafiltration apparatus used was an improved form of that described by Brukner [1926]. About 200 cc. of the dialysed extract were filtered through parchment paper at a pressure of 50 kg. The solid residue was removed, ground up with distilled water and filtered through ordinary filter-paper. This filtrate was again subjected to ultrafiltration, the whole process being repeated a third time, the ultrafiltrates being collected each time. The residue finally obtained was dried in a vacuum desiccator.

Effect of addition of ultrafiltrate on the activity of the enzyme. The effect of addition of the ultrafiltrate on the activity of the enzyme was investigated at 30° and the results are given in Table I, the activity being expressed in cc. thiosulphate.

Table I.

80 cc. of 0.06 % tyrosine solution and 20 cc. of acetate buffer at p_H 6.5 and 10 cc. of toluene were taken in each case.

No.	Enzyme solution	Water or ultrafiltrate	Time (hrs.)		
			2	4	5
I	20 cc. dialysed extract	10 cc. water	4.5	5.2	6.3
II	20 cc. of a solution of ultrafiltered enzyme	"	2.0	3.4	4.0
III	"	10 cc. ultrafiltrate 1	1.6	1.9	3.3
IV	"	10 cc. ultrafiltrate 2	1.4	2.9	3.5
V	"	10 cc. ultrafiltrate 3	1.3	3.1	3.7
VI	"	10 cc. boiled juice	1.8	3.4	4.1

It is clear that the enzyme has not lost its activity on ultrafiltration and that the addition of boiled juice or the ultrafiltrate does not cause any acceleration. On the other hand, slight inhibition is caused, this being greatest with ultrafiltrate 1 and least with 3. The solid content of enzyme solution I (dialysed) was 4 times that of the ultrafiltered enzyme solution but its activity was only 1.5 times as great. Results of experiments done at the same solid content are given in Table II.

Table II.

Time in hours	Activity in cc. thiosulphate	
	Ultrafiltered enzyme	Dialysed extract
1	1.0	0.6
2	1.6	—
4.5	3.3	1.5
7.0	4.5	2.0

The results clearly indicate that the ultrafiltered enzyme is more than twice as active as the dialysed preparation. Conductivity measurements showed that the conductivity of the ultrafiltered enzyme was one-twelfth that of the dialysed preparation. It is therefore evident that ultrafiltration is a good method of purification and that the enzyme is not separated into two components as claimed by Haehn.

Comparison of precipitated enzyme with the dialysed extract. The results are given in Table III.

Table III.

Time in hours	Activity in cc. thiosulphate	
	Precipitated enzyme	Dialysed extract
0.5	4.0	5.2
1.0	8.1	6.4
2.0	10.6	9.5

It is evident that the precipitated enzyme is slightly more active than the dialysed extract.

Electro-osmosis. It has been shown previously [Narayanamurti and Norris, 1928] that cholan malt diastase could be separated into two components by electro-osmosis. Similar experiments were tried with tyrosinase, but it was found that the addition of anode and cathode cell liquids to the middle cell liquid caused only a slight diminution of activity, thus again showing that tyrosinase cannot be separated into two components.

SUMMARY.

Dolichos tyrosinase purified by ultrafiltration is more active than the dialysed preparation. Addition of ultrafiltrate causes no acceleration; on the other hand, slight inhibition is observed.

On repeated precipitation tyrosinase does not lose its activity.

Addition of cathode cell or anode cell liquid to the middle cell liquid of tyrosinase subjected to electro-osmosis in a five-celled apparatus does not cause any increase in activity. Slight inhibition is caused.

All these results clearly indicate that tyrosinase cannot be separated into two components. Any activator present must be in the colloidal condition or bound to a colloidal carrier.

REFERENCES.

- Brukner (1926). *Z. Ver. deut. Zuckerind.* **76**, 419.
 Haehn (1920). *Biochem. Z.* **105**, 169.
 Narayanamurti and Rasmuswami (1929). *J. Indian Inst. Sci.* **12 A**, 109.
 — and Norris (1928). *J. Indian Inst. Sci.* **11 A**, 134.
 Raper and Wormald (1923). *Biochem. J.* **17**, 454.

LXXXIII. THE ACTION OF TANNASE ON GALLOTANNIN.

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(Received March 31st, 1931.)

TANNASE hydrolyses gallotannin with production of gallic acid. The activity of tannase can thus be measured by estimating either the unchanged gallotannin or the gallic acid formed. Micro-methods were therefore elaborated in this laboratory for the estimation of gallotannin [Spiers, 1914; Rhind and Smith, 1922] and gallic acid [Nicholson and Rhind, 1924]. The application of these methods to the tannase problem, using gallotannin freed from glucose [Nierenstein, Spiers and Hadley, 1925], gave an unexpected result, namely, that more gallotannin was hydrolysed than corresponded with the gallic acid produced. It is now shown that this is due to the presence of an enzyme in tannase which destroys gallic acid. This enzyme, for which the name pyrogallase is suggested, is also produced by growing *Aspergillus niger* on media which contain pyrogallol or gallic acid. The presence of pyrogallase in tannase obviously forbids the use of tannase in connection with the chemistry of gallotannin, as was originally planned by us, and this also applies to other tannins which yield gallic acid on hydrolysis [*cf.* Fischer and Bergmann, 1918; Freudenberg, 1919, 1920; Freudenberg and Peters, 1920; Perkin and Uyeda, 1922; Freudenberg and Blümmel, 1924; Freudenberg and Frank, 1927]. The production of pyrogallase by moulds thus affords an explanation of the fact, well-known in industry, that the action of moulds produces appreciably less gallic acid from gallotannin than does acid hydrolysis, although Pottevin [1901] claims a yield of 98 % of gallic acid by the action of *A. niger* on gallotannin.

EXPERIMENTAL.

I. *Cultivation of A. niger.* Czapek's medium as modified by Knudsen [1913] was used; the concentrations of gallotannin, pyrogallol and gallic acid being 4, 2.5 and 0.8 % respectively. The crops were grown in Fernbach flasks or in ordinary large bottles placed on their sides. Each flask held about 350 cc. of medium and each bottle about 150 cc. when exposing a maximum surface. They were plugged tightly with cotton wool, covered with leadfoil and sterilised by intermittent steaming for an hour on three consecutive days. They were then inoculated from the required stock with a sterilised platinum needle. After being well shaken to induce the spores to spread over the surface

of the medium the vessels were placed in an incubator at 23°. After about 4 days the surface was covered generally fairly evenly by a thick white felt, and spores were beginning to form. At this stage the felt was removed, as during the spore formation the fungus excretes enzymes freely into the medium [Dox, 1910]. The felt was removed with a glass rod, washed with tap-water to remove both spores and medium, squeezed as dry as possible, put into a flask and covered with acetone, in which it was allowed to stand overnight. This killed the mycelium, dissolved out the colouring matter and removed the final traces of medium. The following day it was ground up in a mortar with fresh acetone and filtered by suction, the process being repeated until the acetone washings were practically colourless and the mycelium reduced to a fine powder. This was finally washed in a little ether, and after being again filtered by suction was left exposed in a cupboard in an evaporating dish until no smell of ether could be detected. The resulting mouse-grey powder is referred to as "mycelium-powder." It was put into a stoppered bottle and kept in the dark until required.

In a few cases one medium was used to grow more than one crop, but between each crop it was filtered through glass wool and re-sterilised. It was found, however, that although the felt was generally greater in amount in the second crop than in the first, it was less reactive, and in the majority of cases, therefore, only one crop was grown on each medium.

II. *Observations on the behaviour of A. niger during spore formation.* Reference has already been made to the observation recorded by Dox that the fungus excretes enzymes during spore formation. Our results using the methods described by Dox are tabulated below. They confirm Dox in every respect.

Table I.

Enzymes other than tannase and pyrogallase	Substrate used for detection	Mycelium- powder	Cultural solution before sporulation	Cultural solution after sporulation
Oxidase	Guaiacum	-	-	-
Peroxidase	Guaiacol	-	-	-
Laccase	Pyrogallol	-	-	-
Tyrosinase	Tyrosine	-	-	-
Protease	Fibrin	? +	-	-
	Gelatin	-	-	-
	Egg-white	-	-	-
Emulsin	Salicin	+	-	-
	Amygdalin	+	-	+
Diastase	Starch	+++	-	++
Invertase	Sucrose	+++	-	++
Maltase	Maltose	++	-	+
Lactase	Lactose	++	-	+
Raffinase	Raffinose	+	-	+
Amidase	Urea	+	-	+
Hippurase	Hippuric acid	-	-	-
Lipase	Ethyl acetate	++	-	+

III. *Hyper-cultivation.* The term hyper-cultivation is used here to designate the growing of the fungus for several successive asexual generations on gallotannin, a freshly prepared medium being used for each generation.

The results obtained show that it is possible to increase the yield of tannase by this method, and that a physiological change is apparently brought about, which persists even after the fungus is removed from contact with gallotannin. Thus in working with three cultures of *A. niger* and growing each for six asexual generations on gallotannin, the following values, definitely showing an increased tannase production, have been obtained. Taking the activity of the first generation of strain A to be 100 after 24 hours' hydrolysis:

Table II.

Strain A		Strain B		Strain C	
Generation	Activity	Generation	Activity	Generation	Activity
1	100	1	254	1	247
2	113	2	300	2	449
3	88	3	161	3	269
4	167	4	184	4	198
5	137	5	216	5	328
6	155	6	264	6	420
α 136		β 306		γ 435	

In the case of all three cultures an inoculation was made from the fifth generation into a prune-agar medium (which was free from gallotannin). The new generation was again inoculated into a gallotannin-containing medium, and the crops thus produced have been labelled α , β and γ respectively. As will be seen, in each case they showed a greater activity than the first generation had done. This is of importance since it apparently indicates the persistence of the increased tannase production, probably due to a physiological change. As far as could be observed there was no morphological change.

IV. *Preparation of tannase and pyrogallase.* A suspension of 500 g. of mycelium-powder in 2 litres of distilled water containing a little chloroform is shaken for 200 hours and the solution filtered at the pump, the remaining solid being washed 4 times with 100 cc. of water containing a little chloroform. To the filtered solution are added 6 litres of absolute alcohol and the precipitate (40 g.) is collected after standing for 2-3 days. The solid, suspended in 150 cc. of distilled water containing a little chloroform, is shaken for 24 hours and filtered through thick filter-paper, and 2 litres of absolute alcohol are added to the filtrate. The precipitate formed (about 18 g. in the case of tannase and 15 g. in the case of pyrogallase) is collected and again purified by dissolving in 100 cc. of water, shaking for 24 hours, filtering and precipitating with 2 litres of absolute alcohol. In this manner the following average yields were obtained: 9 g. tannase, 6 g. pyrogallase and 2 g. pyrogallase by growing on media containing gallotannin, pyrogallol and gallic acid respectively.

V. *General properties of tannase and pyrogallase.* Both enzymes are completely soluble in cold water. Tested by Dox's methods they are found to give positive reactions for diastase, invertase, maltase, raffinase, amidase and lipase. The pyrogallol nucleus undergoes a profound change when acted on by pyrogallase, it loses its colour-producing properties with iron salts after

some time. The mechanism of this reaction is difficult to explain and all our attempts to isolate some definite disintegration product have led to disappointing results. Table III gives the values observed for the action of 0.1 g. of pyrogallase on 50 mg. of pyrogallol in 100 cc. of distilled water.

Table III.

Exp. 25.		Exp. 34.	
Time hrs.	Pyrogallol found mg.	Time hrs.	Pyrogallol found mg.
24	30.76	48	27.16
72	12.22	72	21.70
96	16.72	93	23.24
144	17.04	147	18.60
168	13.40	174	15.54
192	12.26	226	17.10
216	11.14	248	10.62
240	12.12	336	8.88
164	11.88	357	3.18

It must be noted that in these two experiments two different preparations of pyrogallase were used.

Table IV gives a typical experiment showing the amount of gallic acid produced by tannase from 100 mg. of anhydrous gallotannin. Column A records the amount of gallic acid found, column B the calculated amount of gallic acid from the estimated amount of the unchanged gallotannin.

Table IV.

Exp. 12.		
Time hrs.	A. Gallic acid found mg.	B. Gallic acid calculated mg.
0	1.59	—
24	13.93	19.10
48	19.74	24.80
72	26.16	31.60
144	37.46	40.70

SUMMARY.

It is shown that *Aspergillus niger* produces in addition to tannase an enzyme (pyrogallase) which destroys gallic acid.

REFERENCES.

- Dox (1910). The internal enzymes of *Penicillium* and *Aspergillus* (Washington).
 Fischer and Bergmann (1918). *Ber. deutsch. chem. Ges.* 51, 1804.
 Freudenberg (1919). *Ber. deutsch. chem. Ges.* 52, 177, 1238.
 — (1920). *Ber. deutsch. chem. Ges.* 53, 232, 1728.
 — and Blümmel (1924). *Liebig's Ann.* 440, 45.
 — and Frank (1927). *Liebig's Ann.* 452, 303.
 — and Peters (1920). *Ber. deutsch. chem. Ges.* 53, 953.
 Knudsen (1913). *J. Biol. Chem.* 14, 185.
 Nicholson and Rhind (1924). *Analyst*, 49, 505.
 Nierenstein, Spiers and Hadley (1925). *J. Amer. Chem. Soc.* 57, 1726.
 Perkin and Uyeda (1922). *J. Chem. Soc.* 121, 66.
 Pottevin (1901). *Compt. Rend. Acad. Sci.* 132, 704.
 Rhind and Smith (1922). *Biochem. J.* 16, 1.
 Spiers (1914). *J. Agric. Sci.* 6, 77.

LXXXIV. THE CHEMICAL ASPECT OF THE DRYING OF TIMBER.

2. THE DRYING OF A SOFTWOOD.

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In a previous communication [Campbell and Booth, 1930] an account was given of the chemical changes which accompany the drying of oak wood under various conditions. It was indicated that owing to certain well-defined differences in composition between hardwoods and softwoods these two classes of timber might be expected to react differently to similar conditions of drying. In pursuance of this indication the same experimental procedure which was applied to oak wood has now been applied to a softwood. The material used was the freshly felled mature wood of Silver fir in which there was no differentiation in colour between heartwood and sapwood. After conversion to sawdust the material was ground, as before, to pass an 80 mesh and be retained by a 100 mesh screen. The analytical methods were the same as those used in previous work, and the results throughout are expressed as percentages of the oven-dry weight of the original wood.

EXPERIMENTAL.

The moisture content of the 80–100 mesh material as determined by the standard oven-drying method was 34.58 % and this result was close to that obtained by the xylene method of Schwalbe [Schorger, 1926, pp. 505–506]. The drying experiments were carried out as follows.

(i) *Air-drying.* A weighed sample of the 80–100 mesh wood flour was placed in a glass jar covered with fine linen and left in the laboratory, with occasional mixing, to dry for a period of 200 days. The final moisture content was 13.27 %.

(ii) *Kiln-drying.* A weighed sample of the original wood was placed on a glass dish in a layer $1\frac{1}{2}$ in. deep and the following kiln-drying schedule, which was considered suitable for 2-inch boards, was applied.

The rate of initial warming of the wood flour was 6.7° per hour at a constant humidity of 75 % up to a temperature of 73.9°. The wood was maintained at 73.9° and 75 % relative humidity for 3 hours and then the

Schedule employed in the kiln-seasoning of 80-100 mesh Silver fir.

Temperature °C.	Relative humidity	Duration of treat- ment (days)	Total time (days)	Assumed moisture content %	Remarks
71.1	75	1	1	35-30	Time includes initial warming
71.1	70	1	2	30-25	---
73.9	65	1½	3½	25-20	---
73.9	60	1½	5½	20-15	---
76.7	55	1½	7	15-10	---
79.4	50	1	8	10	Time includes final cooling

temperature was allowed to fall to 71.1°. Final cooling took place at the rate of 6.7° per hour.

As a result of this treatment the moisture content of the wood was reduced to 9.57 %. There was slight darkening in colour and a loss of wood substance amounting to 0.89 % of the oven-dry weight of the original wood was recorded.

(iii) *Oven-drying.* (a) A weighed sample of the original wood was maintained at 105° for 16 hours. The material became darker in colour.

(b) A similar sample of the original wood was maintained at 105° for 9 days. There was pronounced darkening in colour and a loss of wood substance amounting to 0.62 % of the oven-dry weight of the original wood was recorded.

The analysis of the original wood and the analyses of the same material after the several drying treatments are recorded in Table I. The data in Table II were obtained in order to determine the effect of 1 % NaOH on the original wood and on the wood after drying.

Table I. *Analysis of 80-100 mesh Silver fir wood before and after air-, kiln- and oven-drying.*

Material	Dura- tion of treat- ment (days)	Loss due to treat- ment	Cold water- soluble	Hot water- soluble	Cellu- lose	Lignin	Meth- oxyl	Total pento- sans	Pento- sans not in cellu- lose	Pento- sans in cellu- lose
Original wood	—	—	1.34	2.57	57.89	28.09	5.29	10.15	6.89	3.26
Air-dried	200	Nil	1.07	2.06	58.64	28.88	4.89	9.44	6.31	3.13
Kiln-dried	8	0.89	1.36	2.82	57.59	28.24	4.67	9.45	6.35	3.11
Oven-dried (a)	½	Not deter- mined	1.60	2.94	57.41	28.27	4.78	9.49	6.41	3.08
(b)	9	0.62	2.35	4.52	57.23	28.19	4.74	9.29	6.34	2.95

Table II. *Analysis of air-, kiln- and oven-dried Silver fir wood after alkali-extraction.*

Material	Loss due to treatment	Cellulose	Lignin	Methoxyl	Total pentosans	Pentosans not in cellulose	Pentosans in cellulose
Original wood	11.14	55.98	26.52	4.01	8.59	4.97	3.62
Air-dried	12.63	55.64	26.41	3.96	7.88	4.70	3.18
Kiln-dried	10.82	55.96	26.37	4.25	8.18	4.84	3.34
Oven-dried (a)	12.22	54.37	26.12	3.66	7.87	4.99	2.88
(b)	15.07	53.38	25.30	3.93	7.27	4.37	2.90

DISCUSSION.

Having due regard to manifest imperfections in analytical procedure, the data in Table I would, at first sight, suggest that during drying Silver fir wood suffers no radical change in composition. Critical examination reveals, however, that the data are consistent with slight though none the less definite chemical changes. For instance, in the case of air-drying, it can be seen that the yields of Cross and Bevan cellulose and of lignin are higher in dry wood than in green wood. These slight increases are accompanied by depreciations of water-soluble material and furfuraldehyde-yielding complexes respectively. Further, the loss in total pentosans represents a combined loss of pentosans in the cellulose and pentosans not in the cellulose. Since no loss in weight was observed, the depreciation in pentosans must be reflected in the increase in lignin content of the dry wood, and it must follow that the source of the accretions to the cellulose must be some material in green wood which is soluble in water. This contention derives support from the fact that as a result of air-drying, the combined losses of water-soluble material and total pentosans represent 1.49 %, and the combined additions to the cellulose and lignin amount to 1.54 % of the oven-dry weight of the original wood.

Further examination of the data in Table I shows that kiln-drying brings about a loss of wood substance which amounts to 0.89 % of the oven-dry weight of the original wood. It is significant that while no additions have been made to the cellulose, the kiln-dried wood contains approximately the same amount of water-soluble material as the original wood. The observed increase in lignin content during drying is extremely small, but it is consistent with previous work. It is interesting to note that the percentage increase in lignin content is exactly equal to the decrease in the pentosan content of the cellulose, and the suggestion thus presents itself that the drying treatment has been responsible for the decomposition of a portion of the furfuraldehyde-yielding complexes which was not associated with the cellulose in the original wood, and which under other conditions of drying might also have become associated with the lignin. This loss in furfuraldehyde-yielding material calculated as pentosans by means of Kröber's table amounts to 0.55 % of the oven-dry weight of the original wood, and thus accounts in large measure for the observed loss in weight.

For obvious reasons it has been impossible to determine directly whether the oven-drying of wood for a comparatively short period results in a loss of wood substance, but indirect evidence on this important question is afforded by the data in Table I. Oven-drying of green Silver fir wood at 105° for 16 hours results in slight, though perceptible, increases in water-soluble material and in lignin content, which are practically offset by corresponding losses in cellulose and furfuraldehyde-yielding complexes. The combined increases amount to 0.81 % and the combined losses to 0.97 % of the oven-dry weight of the original wood. The loss in weight during drying has thus been

exceedingly small. It is significant that here again the slight increase in lignin content is exactly equal to the decrease in the pentosan content of the cellulose. It would thus appear that the drying treatment has again precluded the permanent addition to the lignin of a further portion of the furfuraldehyde-yielding complexes which in the green wood was not associated with the cellulose.

The experimental data do not reveal the effect of oven-drying at 105° on wood which has already been air-dried, but if it is assumed that on oven-drying for the period commonly adopted in moisture content determinations air-dried wood would attain the same composition as green wood heated under the same conditions, it can be seen from Table I that the loss of wood substance during drying would not exceed 0.43 % of the oven-dry weight of the original wood.

When Silver fir wood is oven-dried for 9 days it is obvious from the data in Table I that incipient decomposition takes place. In the first place there is a loss of wood substance, and secondly, the dry wood contains a much higher proportion of water-soluble material than the original wood. There is a slight depreciation of cellulose and the lignin and pentosan contents are lower in the oven-dried wood than in any of the other samples.

Turning now to the data in Table II it can be seen that an interesting comparison is afforded between the alkali-solubility of the original wood and the alkali-solubility of the same material after the various drying treatments. Further, a comparison of the data in Tables I and II shows the effect of 1 % NaOH at 100° on the major wood components both before and after drying. The most striking fact illustrated by Table II is that after alkali-extraction the original, air-dried, and kiln-dried wood are approximately of the same composition, despite the fact that the alkali-solubility of the air-dried wood is greater than that of either the original or the kiln-dried wood. While alkali attacks the pentosans not in the cellulose of all the samples, the pentosan content of the cellulose exhibits a slight though perceptible increase in amount in each case. The cellulose and lignin aggregates in air-dried wood are more soluble in alkali than the corresponding aggregates in either green or kiln-dried wood. The only explanation of this fact would appear to be that the material added to these aggregates during air-drying is readily soluble in alkali. Since addition to the cellulose does not take place during kiln-drying the alkali-solubility of this component is not increased, but the alkali-solubility of the lignin is slightly increased because in this case material is added during drying. The increase in alkali-solubility of the lignin is offset by a decrease in the alkali-solubility of the pentosans not in the cellulose.

The relatively high alkali-solubility of the oven-dried wood can only be explained by the fact that incipient hydrolysis has taken place as a direct result of the high temperature treatment. This was indicated in Table I by the higher water-solubility of oven-dried wood, but the results in Table II reveal that, irrespective of the fact that after 16 hours' drying at 105° no

additions were made to the cellulose, the alkali-solubility of this component has increased. The incipient hydrolysis has apparently been confined to the carbohydrate components of the wood substance.

Prolonged oven-drying of green wood results in a pronounced increase in the alkali-solubility of the wood as a whole, which is reflected in the greater susceptibility to the reagent of the lignin as well as the carbohydrate components. In the light of previous work [Hawley and Campbell, 1927] the effect of prolonged oven-drying can only be described as a partial hydrolysis.

CONCLUSIONS.

From a comparison of the foregoing data with those obtained in the study of the drying of oak wood [Campbell and Booth, 1930] it is to be concluded that although hardwood and softwood react somewhat differently to similar drying conditions, they have one feature in common. As moisture leaves the wood, changes take place in the furfuraldehyde-yielding complexes of the wood substance. Under suitable conditions of drying the hardwood cellulose and lignin respectively are enhanced at the expense of the furfuraldehyde-yielding complexes, whereas under similar conditions only the lignin of the softwood is enhanced from this source. It is to be concluded that the material added to the cellulose of the softwood during air-drying is probably of an unstable nature, unlike true cellulose in composition, since it is recruited from water-soluble material in the green wood, and can readily be split off by dilute alkali from the air-dried wood.

In general, for both types of wood the effect of moderate heat during drying is to reduce the amount of the additions to the cellulose and lignin, while high temperatures definitely cause hydrolysis. It has become apparent from the results of research at present in progress that even the incipient hydrolysis of wood substance caused by fungi of the brown rot type has such marked effects on the physical properties of timber as the decrease of mechanical strength and a tendency to cause brittleness. Thus it becomes obvious that the woods which are chemically best suited to withstand high temperatures during drying are those which are least susceptible to hydrolysis. It has been shown in previous work that the pentosans, or rather the furfuraldehyde-yielding complexes of wood substance, are the most susceptible to hydrolysis of all the principal wood components and the suggestion has been made [Campbell and Booth, 1930] that a wood of high pentosan content would be more susceptible to heat than a wood of low pentosan content. The results of the present investigation would appear to bear this out.

In the first place, by comparison with previous data [Campbell and Booth, 1930] it can be seen that the extent of the depletion of the pentosans *per se* during drying is approximately of the same order of magnitude in the hardwood and softwood, although depletion in the hardwood takes place only in the pentosans not in the cellulose. Since, however, the softwood contains less than half the percentage of pentosans present in the hardwood, the net effect

of drying on the former is correspondingly less pronounced than it is on the latter.

Purely physical considerations render it difficult in practice to remove the greater part of the moisture from green wood without at the same time producing some of the defects commonly described in technical literature, but since rapid drying is so frequently effected by employing heat and high relative humidities, the question arises as to whether some defects may, in part, be due to chemical changes induced by unsuitable conditions of drying. For instance, it has been pointed out by Stillwell [1928] that all species of wood are not equally tolerant of heat, and the suggestion is further made that there may be a temperature for all species above which seasoning cannot proceed without damage. At the same time it is eminently feasible that there may be critical temperatures for individual species above which drying cannot proceed without causing a degree of hydrolysis which will be reflected in decreased strength of the dry wood. In this connection it should be borne in mind that apparently small changes in composition detected by current methods of analysis may cause marked changes in physical properties, and the results of the present series of investigations suggest that, since the chemical changes due to the drying of timber originate in small changes in the furfuraldehyde-yielding complexes of the wood substance, the so-called pentosans of wood substance play an important part in the determination of physical characteristics of the material as a whole.

Note on the determination of moisture by the standard oven-drying method.

The present investigation throws a new light on the question of moisture determination by the oven-drying method. It is clear that oven-dried wood is not exactly of the same composition as green wood. After drying for 16 hours at 105° the error in moisture content in the case of green Silver fir wood can amount to 0.16 %. For air-dried wood the error might be appreciably greater, and it is conceivable that the degree of error might vary from species to species. The degree of error estimated above cannot in itself be regarded as serious, but, at the same time, it would appear to be advisable in determining moisture by the oven-drying method not to prolong the drying time longer than is absolutely necessary for the samples to attain constant weight. In this way error due to incipient hydrolysis would be minimised.

The effect of 1 % NaOH at 100° on wood substance.

It was reported in a previous paper [Campbell and Booth, 1930] that after oak wood had been heated at 100° with 1 % NaOH the pentosan content of the cellulose was higher than that of the untreated wood. A similar result was later obtained with beech wood [Campbell, 1930]. This was thought to be a characteristic reaction of hardwoods as it had never been reported for a softwood. The same phenomenon has been observed, however, in the present investigation in the wood of Silver fir, although the increase in the pentosan

content of the cellulose here is by no means so great as in the hardwoods examined. These results suggest a possible difference in composition between the furfuraldehyde-yielding complexes which are associated with the cellulose of wood on the one hand and those which are not associated with the cellulose on the other. The matter will receive more detailed investigation.

SUMMARY.

1. The chemical effects of air-, kiln- and oven-drying on the wood of Silver fir have been examined in detail.
2. Air-drying is shown to result in depreciation of pentosans and water-soluble material. Lignin is enhanced at the expense of the furfuraldehyde-yielding complexes, and cellulose is enhanced at the expense of the water-soluble material of the green wood.
3. Kiln-drying precludes the additions to cellulose and part of the additions to lignin.
4. Oven-drying of green wood induces incipient hydrolysis of the wood substance.
5. An attempt has been made to confirm a previous suggestion that softwoods withstand high temperatures during drying better than hardwoods on account of their relatively low pentosan content.
6. The oven-drying method of moisture determination is subject to a slight error due to incipient hydrolysis of wood substance.

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REFERENCES.

- Campbell (1930). *Biochem. J.* **24**, 1235.
— and Booth (1930). *Biochem. J.* **24**, 641.
Hawley and Campbell (1927). *Ind. Eng. Chem.* **19**, 742.
Schorger (1926). *Chemistry of cellulose and wood* (McGraw Hill Book Co.).
Stillwell (1928). *Forest Products Research Laboratory Leaflet*, No. 2.

LXXXV. A STUDY OF THE HYDROGEN ION CONCENTRATION OF THE POTATO TUBER.

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NUMEROUS investigations have been made on the acidity of plant sap and several methods have been employed. The hydrogen electrode has been used extensively but the time required for the system to reach equilibrium, the alteration of the biological fluid by the passage of hydrogen and the poisoning of the electrode by the plant products are obvious disadvantages of the method. Colorimetric methods are not entirely satisfactory on account of the rapid colour changes which may take place in the sap as a result of enzyme action. With the quinhydrone electrode, the precision of which has been critically examined by Morgan, Lammert and Campbell [1931], equilibrium is attained very rapidly and manipulation is simple. Plant oxidases, however, attack quinol [Chodat, 1910] and, consequently, the ratio quinol:quinone is liable to be altered. In this paper, the results obtained by rapid measurement of the acidity of tuber tissue with a special quinhydrone capillary electrode are described and compared with those obtained by other methods.

METHODS.

(a) *Extraction of sap.* When it was desired to examine the sap, the tubers were washed in cold water and passed through a pulping machine. The mash was filtered through fine linen and the filtrate, which gave essentially the same results as the pulp, was generally employed. The pulped tubers decomposed rapidly so that the sap usually had a red-brown colour which became darker on standing.

(b) *Hydrogen electrode measurements.* A Hildebrand half-cell was used in conjunction with a saturated calomel electrode. About 5 cc. of the plant sap were taken for each determination and equilibrium was reached after approximately 10 minutes. The hydrogen electrode was given a fresh deposit of platinum black after each measurement.

(c) *Quinhydrone electrode measurements.* The quinhydrone electrode consisted of a piece of polished platinum foil which was placed in the potato sap saturated with quinhydrone. Some preliminary experiments showed that at least 0.12 g. of quinhydrone had to be added to each 10 cc. of sap in order to

obtain reproducible results. The procedure adopted was to shake the sap with sufficient quinhydrone for a few seconds, complete the potentiometer circuit with the platinum and saturated calomel electrodes and read the E.M.F. of the cell at once. The whole operation was carried out in less than 1 minute but decomposition of the phenol had already commenced. The platinum foil was washed and heated to redness in an alcohol flame after each measurement and from time to time was also cleaned in hot chromic acid solution.

A comparison of results obtained by methods (b) and (c), included in Table III, shows that in most cases the hydrogen electrode yields slightly higher p_H values. This may be due to some alteration in the sap during the passage of hydrogen or to some error in the application of the quinhydrone half-cell. The fact that the sap, if allowed to stand for about 10 minutes before the addition of the quinhydrone, gave approximately the same value as with the hydrogen electrode, suggests that the time required by the system to reach equilibrium is the important factor.

(d) *Micro-electrode measurements.* Reproducible results were readily obtained with the quinhydrone electrode, but the use of pulp in any method removes the possibility of studying the variation in acidity throughout the tuber. To meet this difficulty a special electrode was constructed [Robertson and Smith, 1930], which enabled measurements to be made at any desired point on a section of the plant tissue. A few results obtained by means of this electrode and by the method described under (c) are given in Table IV. The p_H of the sap was always greater than that observed with the micro-electrode for the tissue, which was probably due to loss of carbon dioxide in the preparation of the pulp. Ingold [1929] observed a reduction of 0.3 p_H unit when the tuber sap was brought into equilibrium with concentrations of carbon dioxide such as have been recorded for the intercellular spaces of the tuber. In addition, the oxidase activity of the disintegrated tissue is very great and the formation of melanin and its intermediate compounds from tyrosine [Raper, 1928] may also reduce the acidity.

Variation in acidity throughout the tuber.

Although some data are available on the hydrogen ion concentration gradients of the sap of plants [Gustafson, 1924], no such observations seem to have been made on the potato tuber. Attention was directed to a study of the changes which take place during the formation, storage and sprouting of the tuber.

(a) *During growth period.* A number of healthy and typical plants was selected and examined at intervals throughout the summer. The tubers were cut along a plane through an eye and the heel. Readings were taken with the micro-electrode at points in the middle and 1-2 mm. below the eye and heel. The results for two varieties given in Table I are typical of a large number of observations and each figure is an average value for four tubers.

Table I. *Variation of tuber p_H with stage of growth.*

Variety	Date of sampling ...	30. vi. 30	21. vii. 30	16. viii. 30	30. ix. 30
Duke of York	"Rose" end	6.19	6.04	5.90	5.65
	Centre	6.20	6.08	5.75	5.71
	"Heel" end	5.53	5.90	5.85	5.73
Epicure	"Rose" end	6.08	6.13	5.81	5.68
	Centre	6.06	6.09	5.88	5.75
	"Heel" end	5.78	5.82	5.80	5.80

The first readings were made with tubers weighing 5–15 g. and it was observed that the underground stem attaching the tuber to the parent plant had approximately the same p_H as the heel of the tuber. The final sampling was made after the plants were completely ripe, the haulms having withered away some time previously. The results show that all parts of the tuber, except the heel, become more acid as the plant ripens. The p_H of the heel rises quickly and then falls off gradually. There is a p_H gradient across the young tuber, the heel being more acid than the rose end. In the mature tuber the reverse holds good. This change coincides with the physiological development of the tuber, for in the early stages of growth the active part is at the heel where nutrients are received from the plant, whereas in the mature state this process has ceased and the eyes develop rapidly.

(b) *During rest period.* A large number of tubers was examined between the time of harvesting and the appearance of sprouts. Observations were made at eleven points on a section of each tuber, both cortex and medulla being represented. In the majority of cases the rose end was slightly more acid than the heel end, the centre being intermediate. The values given in Table I for the last sampling are typical in this respect. In many tubers, however, no difference was noted between the rose and heel ends, while in a few isolated cases the heel was slightly more acid than the rose end. Generally, the average value for all the points approximated closely to that for the centre of the tuber.

(c) *During sprouting period.* In March a number of dormant tubers was exposed to daylight and allowed to sprout. At certain intervals a few tubers were cut down through a sprout and eye to the heel and the acidity was determined at a number of positions across the section. Tubers of nine varieties were examined and all yielded similar results. In Table II the average values for three tubers of the variety Duke of York are given.

Table II. *Variation of p_H during sprout formation.*

Position on tuber or sprout	20. iii. 30	16. iv. 30	15. v. 30	24. vi. 30
Growing tip of sprout	4.28	4.30	4.38	4.51
Middle of sprout	4.45	4.55	4.62	4.90
Tuber end of sprout	4.25	4.36	4.54	5.09
Surface of "eye"	4.58	4.73	5.70	5.85
2 mm. below surface of "eye"	5.31	5.60	5.86	—
5 mm. below surface of "eye"	5.61	5.67	5.83	—
10 mm. below surface of "eye"	5.75	5.74	5.86	—
Middle of tuber	5.74	5.71	5.78	5.80
"Heel" end of tuber	5.80	5.85	5.86	5.88

The following is a description of the sprouts:

- 20. iii. 30.—Sprouts soft and light green, and about 0.5 cm. long.
- 16. iv. 30.—Sprouts hard and dark green, 0.5–1.5 cm. long.
- 15. v. 30.—Sprouts thicker, 2.0–2.5 cm. long, small leaves forming.
- 24. vi. 30.—Sprouts very thick and hard, 3 cm. long, small leaves and shoots formed. Tuber soft.

When sprouts appear, the region round the active eyes is very acid, but gradually becomes less acid as the sprouting progresses until June, when the p_H is practically uniform throughout the tuber. The growing tip of the sprout retains its relatively high acidity throughout the growth of the plant; in a young field plant it had p_H 4.46 while in an older plant the p_H was 4.65.

These variations in p_H through the tuber are to be expected if consideration is given to the different functions of the various parts. The actively growing parts are usually more acid than the fully developed members. On the other hand, the acidity of the tuber increases with maturity, which may be accounted for by the fact that, according to Appleman and Miller [1926], the protein in the young tuber gives place to non-protein- and amino-nitrogen in the mature tuber.

Factors influencing the p_H value of the mature tuber.

(a) *Environment.* Tubers of six common varieties, grown at seven places in Midlothian, were examined immediately after harvesting. The altitudes of the different places varied from 100 to 600 feet above sea level; the different soils varied in texture from sandy loams to clay loams and in p_H from 4.9 to 7.3. There was no obvious relationship, however, between environment and the acidity of the tuber. The maximum variations of individual values for all sources were as follows: Duke of York, 5.70–5.89; Epicure, 5.70–5.85; Great Scot, 5.65–6.11; Golden Wonder, 5.70–5.85; Majestic, 5.50–5.73; Ally, 5.67–5.84.

In order to investigate more fully the effect of soil reaction upon the acidity of the tuber, a crop of variety King George, grown on a series of specially treated plots on a soil of p_H 4.0, was examined. The p_H of the tubers was measured by methods (b) and (c) and the results are summarised in Table III.

Table III. *Relation between p_H of soil and tuber sap.*

p_H of soil	No. of plots	p_H of tuber sap	
		Method (b)	Method (c)
4.0–4.5	4	5.69	5.66
4.5–5.0	11	5.68	5.64
5.0–5.5	11	5.66	5.63
5.5–6.0	5	5.69	5.63
6.0–6.5	3	5.68	5.63
6.5–7.0	2	5.62	5.68

In 1929, five plots were laid down on a soil having p_H 5.5. Two plots were made more acid by treatment with flowers of sulphur and two more alkaline by treatment with calcium hydroxide. Two varieties were grown on each plot.

In autumn, samples of tubers were lifted, washed and dried and then examined as follows. Ten tubers were selected at random from each sample and cut through the rose and heel ends. The acidity was determined at various points on the section by means of the micro-electrode. The small injuries made by the electrode were then cut out, the tubers were pulped and the p_H of the sap was determined by method (c). The average results are given in Table IV, the p_H values of the soil being those obtained during September.

Table IV. *Relation between p_H of soil and tuber.*

Plot	p_H of soil	Average p_H of tubers			
		Duke of York		Great Scot	
		(d)	(c)	(d)	(c)
A	8.1	5.70	5.77	5.68	5.73
B	7.5	5.68	5.75	5.69	5.73
C	5.5	5.70	5.74	5.69	5.70
D	5.1	5.70	5.73	5.68	5.74
E	5.0	5.71	5.77	5.68	5.72

It is obvious from the above results that the p_H of the tuber is not influenced by that of the soil. Hoagland and Davis [1925] have obtained similar results for other plants grown in culture solutions, but other workers, for example Haas [1920], have found a certain relationship between the acidities of soil and plant sap.

(b) *Variety*. In addition to the p_H figures already given for different varieties, the following were obtained: Arran Consul, 5.64; Field Marshal, 5.74; Kerr's Pink, 5.75; King Edward, 5.95. The differences between varieties were thus not significantly greater than the variations found among tubers of the same variety.

(c) *Disease*. A number of tubers affected by various diseases was collected and compared with normal tubers of the same variety. The influence of virus infection was also studied. The results are presented in Table V.

Table V. *The effect of disease upon the p_H of tubers.*

Variety	Nature of infection	Normal tuber	Affected tuber	
			Healthy part	Diseased part
Great Scot	Corky scab: <i>Spongospora subterranea</i> Lagerh.	5.70	5.65	4.35
Duke of York	Common scab: <i>Actinomyces scabies</i> (Thaxt.) Güssow	5.75	5.70	4.58
Epicure	Sprain: <i>Bacterium rubefaciens</i>	5.73	—	5.60
Epicure	Blackleg: <i>Bacillus atrosepcticus</i> van Hall	5.73	5.68	5.65
Duke of York	Blight (fresh): <i>Phytophthora infestans</i> (Mont.) De By.	5.75	5.75	5.83
Duke of York	Blight (old)	5.75	5.70	5.38
Duke of York	Wart disease: <i>Synchytrium endobioticum</i> (Schilb.) Perceval	5.75	5.65	5.02
Ally	Mosaic	5.80	5.70	
	Crinkle	5.80	5.60	
	Leaf Roll	5.80	5.85	
Arran Comrade	Mosaic	5.64	5.47	
	Leaf Roll	5.64	5.70	

In diseased tubers a very acid region was observed around the affected part. In mild forms the disease did not seem to affect the rest of the tuber but in severe cases a slight increase in acidity was noted throughout the tuber. The slight decrease in acidity due to Leaf Roll and the large increase due to Wart Disease confirm results reported by Boas [1919] and Weiss and Harvey [1921] respectively. Severe forms of mosaic, such as crinkle, gave rise to an increase in acidity.

(d) *Storage*. A number of tubers of each of six varieties was stored in three different ways, *viz.*: (1) in a pit in the open, (2) in a cool, well-ventilated storehouse, (3) in a refrigerator maintained at 2°. After a few months the average p_H values were as follows:

Original value (October)	5.76
After storage (2)	5.84
„ (1) immediately	5.49
„ (1) after 24 hours at room temperature					5.88
„ (3) immediately	5.39
„ (3) after 24 hours at room temperature					5.83

The tubers from the pit and refrigerator were more acid than before storage, but, on being allowed to stand at room temperature for a day, acquired a p_H slightly higher than the original. This was probably due to increased respiratory activity of the tissue immediately after removal from such storage conditions.

SUMMARY.

The hydrogen and quinhydrone electrodes have been used for p_H measurements of the pulp of the potato (*Solanum tuberosum*), and a micro-electrode enabled measurements to be made at different points of the tissue.

The acidity is not uniform throughout the tuber but depends upon the function of the different parts at different periods in the life of the plant. In the early stages of development the underground stem and the heel end of the tuber are most acid and the average acidity of the tuber increases with maturity. In the dormant state the acidity does not vary much in different parts of the tuber but when sprouting begins the active eyes are most acid.

The acidity of the tuber is independent of environment and is not influenced by large variations in soil acidity.

The differences in acidity due to variety and storage are not significant.

Comparatively large changes in acidity are associated with certain diseases.

REFERENCES.

- Appleman and Miller (1926). *J. Agric. Res.* **33**, 569.
Boas (1919). *Z. Pflanzenkrankh.* **29**, 171.
Chodat (1910). Abderhalden's Biochem. Arbeitsmethoden, **3**, 42.
Gustafson (1924). *Amer. J. Bot.* **11**, 1.
Haas (1920). *Soil Sci.* **9**, 341.
Hoagland and Davis (1925). *New Phyt.* **24**, 99.
Ingold (1929). *Protoplasma*, **6**, 51.
Morgan, Lammert and Campbell (1931). *J. Amer. Chem. Soc.* **53**, 454.
Raper (1928). *Physiol. Rev.* **8**, 245.
Robertson and Smith (1930). *J. Soc. Chem. Ind.* **49**, 120 T.
Weiss and Harvey (1921). *J. Agric. Res.* **21**, 589.

LXXXVI. A NOTE ON THE INHIBITORY EFFECT OF MONOiodoacetic acid ON LACTIC ACID PRODUCTION BY CANCER TISSUE.

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(Received April 6th, 1931.)

LUNDGAARD, in recent work [1930, 1] has shown that monoiodoacetic acid prevents the production of lactic acid in contracting muscle. Later [1930, 2] he found that, with concentrations of iodoacetic acid which completely inhibit glycolysis, the oxidising systems of yeast and muscle remain unimpaired. Warburg has shown that tumour cells derive a large part of their energy for growth from glycolysis. If this glycolysis of tumour could be inhibited by iodoacetic acid as is that of muscle, the growth of the tumour might be checked. Lundsgaard states that he is testing the effect of iodoacetic acid on the growth of tumours in mice by injecting it into the living animal. In order to see whether such inhibition of growth might be expected in tumours, we tried the effect of iodoacetic acid on glycolysis of tumours *in vitro*. So far as is known, the mechanism of glycolysis in tumours differs considerably from that in muscle, the tumour glycolysis appearing to be independent of hexosephosphate [Harrison and Mellanby, 1930]. Lundsgaard showed that in muscle under the influence of iodoacetic acid hexosephosphate is synthesised but not broken down. Since the production of lactic acid in the tumour cell appears to be independent of the breakdown of hexosephosphate, it seemed to us possible that iodoacetic acid might not produce the same inhibiting effect upon the lactic acid production by tumours as it produced upon that by muscle. The experiments given in the table show, however, that iodoacetic acid causes a large inhibition in the production of lactic acid by tumours *in vitro*. As yet we are unable to explain by what mechanism the iodoacetic acid causes such inhibition in tumour glycolysis.

EXPERIMENTS *IN VITRO*.

Mouse carcinoma 63 was cut into thin slices, weighed and dropped into flasks containing 50 cc. bicarbonate Ringer's solution (brought to p_H 7.8 by passing through it a mixture of 5 % CO_2 and air), 5 cc. 9.6 % glucose (final concentration = 0.8 %), and 5 cc. either of water or of a neutralised solution of iodoacetic acid dissolved in 0.8 % NaCl. 30 cc. were then removed into a solution of trichloroacetic acid for the determination of the amount of lactic

acid already present¹. The flasks were then stoppered and shaken for 3 hours at 37° and, after removal of proteins and carbohydrates, the lactic acid was estimated by the method of Friedemann, Cotonio and Shaffer [1927].

Table I. *Aerobic glycolysis of tumour tissue as affected by iodoacetic acid.*

					mg. lactic acid per g. dry weight tissue mg.
Exp. 1.	Control	39.1
	Iodoacetic acid 2 mg. in 30 cc.				14.5
Exp. 2.	Control	41.4
	Iodoacetic acid 4 mg. in 30 cc.				7.3
Exp. 3.	Control	34.6
	Iodoacetic acid 4 mg. in 30 cc.				1.0
Exp. 4.	Control	30.3
	Iodoacetic acid 1 mg. in 30 cc.				8.9
	.. 2 mg. in 30 cc.				5.6
	.. 4 mg. in 30 cc.				3.0

In Exp. 3. the tissue had been shaken for an hour in bicarbonate Ringer's solution and then put into fresh Ringer's solution with glucose and shaken with and without iodoacetic acid.

EXPERIMENTS IN VIVO.

Several experiments were done in which iodoacetic acid was injected in a sublethal dose into mice bearing tumour 63. After a given time, the animals were killed and the glycolysis of the tumour compared with that of tumours from uninjected animals. In order to make the control as valid as possible, both the injected and uninjected animals bore tumours from the same inoculation. Since there is some variation in the glycolytic values of untreated cancerous tissues from different animals even when the tumours are produced from the same parent tumour, it is evident that only very large differences between the degree of glycolysis of the tumours of the injected and the uninjected animals would be significant. Glycolytic values from a number of tumours were compared to give the control figure. The glycolytic value of 5 tumours of the same generation ranged between 31 and 43 mg. lactic acid per g. of tissue, and an average value of 37 mg. was taken for the control figure. The animals were given a subcutaneous injection (on the side opposite the tumour) of 0.2 cc. of 1 % iodoacetic acid (previously neutralised) and were killed from 1 to 2 hours afterwards. Their tumours were removed, sliced and put into sugar-containing bicarbonate Ringer's solution, and the experiment was run as already described. Animals A and B, killed respectively 1 and 2 hours after injection of iodoacetic acid, appeared to be normal and showed no symptoms of iodoacetic acid poisoning. Animal C, on the other hand, was almost moribund when killed 2 hours after injection.

¹ In the figures given in the tables this amount of preformed lactic acid has already been subtracted from the values both for the control solutions and those containing iodoacetic acid.

Table II. *Aerobic glycolysis of tumour slices after injection of iodoacetic acid.*

					Lactic acid per g. dry weight tissue mg.
Animal A	48.8
Animal B	56.5
Animal C	12.9
Control	37.0

The figures for the tumour glycolysis of the injected animals are an average of three experiments done on each tumour.

These results, although varying considerably from the control, at least indicate that when the dose of iodoacetic acid is sublethal, there is no inhibitory effect upon lactic acid production of the tumour after a single injection, but when the dose causes acute symptoms of poisoning, the lactic acid production of the tumour is strongly inhibited.

SUMMARY.

1. The aerobic production of lactic acid by tumour slices is inhibited by iodoacetic acid.

2. A few experiments, on mice, in which a single injection of iodoacetic acid was given, show that no inhibition of lactic acid production in tumour occurs provided that the dose is sublethal.

We are indebted to the Yorkshire Cancer Research Fund for the expenses incurred in carrying out this work.

REFERENCES.

- Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.
 Harrison and Mellanby (1930). *Biochem. J.* **24**, 141.
 Lundsgaard (1930, 1). *Biochem. Z.* **217**, 162.
 ——— (1930, 2). *Biochem. Z.* **220**, 8.

LXXXVII. THE BLACKENING OF POTATOES AFTER COOKING.

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It is well known that potatoes after cooking sometimes darken considerably on the surface. This blackening is distinct from the darkening which occurs with raw potato on exposure to air. In the latter case, there is no doubt that the darkening is due to enzymic oxidation, the oxidisable substance being a phenolic substance of the nature of catechol or other *o*-dihydroxybenzene derivative [Onslow, 1919, 1920] or tyrosine [Bertrand, 1896; Gallagher, 1923].

The darkening of potatoes after cooking appears to be a matter of some considerable importance to those concerned in the growing and sale of potatoes. At the request of the Ministry of Agriculture some experiments were carried out in the Household Arts Department of this College with reference to methods of cooking potatoes in order to find out whether such darkening could be avoided. Some of the potatoes supplied by the Ministry of Agriculture were obtained by the author in order that the problem might be investigated from a chemical standpoint.

In the first experiments "King Edward" and "Great Scot" potatoes were employed and later "Majestic" potatoes were used. Several batches of potatoes of these three varieties have been used in the later experiments, but in the experiments to be described in this paper the potatoes which do not blacken on cooking will be referred to as potatoes A, and those which blacken as potatoes B.

One of the first objects of the present investigation was to devise, if possible, a simple test by means of which it would be possible to tell by examination of a raw potato whether or not a similar potato would blacken on cooking. It is believed that such a test has been discovered. It is described below under the action of nitrous acid on potatoes.

In preliminary experiments, tests were made with various reagents to ascertain if any great difference could be detected between those potatoes which blacken after cooking and those which do not.

Enzymic oxidation.

Transverse sections about 5 mm. thick of potatoes A and B were peeled thinly and exposed to air for one day (in some cases two days) in a bell-jar

containing some chloroform and some water. In both cases very dark brown colours were produced on the surfaces exposed as shown in Plate III, Figs. 1 and 2. Thus enzymic oxidation shows no difference between the two varieties.

The action of nitrous acid.

It was found that all potatoes examined contain a substance (or substances) in greatly varying amounts, which on treatment with nitrous acid followed by an alkali, gives a fine red colour. The amount of the red substance produced in this test was found to vary exactly with the amount of blackening which takes place on cooking. The test is carried out as follows. A transverse section of potato about 5 mm. thick is peeled thinly and covered with 7 % sodium nitrite solution (about 25 cc.) in a small porcelain basin. About 2 cc. of dilute hydrochloric acid (1 volume of concentrated hydrochloric acid to 2 volumes of water) are added and the mixture left for 5 minutes. The liquid is then poured off and the section of potato covered with 16 % sodium hydroxide solution (about 25 cc.). The red colour develops in about 5 minutes, at first chiefly on the outer and inner edges of the fibro-vascular layer of the potato, then through the whole of this layer, but it often extends towards the centre. It is strongly marked where there are eyes in any potato. After some time the coloured substance is partly extracted by the sodium hydroxide solution.

This reaction has not yet been investigated fully. It may be that the nitrous acid reacts with a primary amino-compound and that coupling of the diazo-compound thus produced takes place with a phenoxide on the addition of the sodium hydroxide. The colour may, however, be due partly or entirely to reactions between the nitrous acid and lignocellulose.

The red colour of the sodium hydroxide solution of the substance produced in the reaction is changed on addition of dilute hydrochloric acid and restored on making alkaline. With concentrated hydrochloric acid, on heating, the red substance is decomposed, as the colour is not restored on again making the solution alkaline.

It appears very probable that the production of this colour in the test described above is in some way connected with the blackening which takes place after cooking. If for example the fibro-vascular layer of a potato B, which gives most colour in the test, is completely removed before cooking very little blackening of the remainder is usually noted after cooking.

Photographs of sections of potato after applying the nitrous acid test are shown in Plate III, Fig. 3, for potato A which does not blacken after cooking, and in Plate III, Fig. 4, for potato B, which blackens.

In Plate III, Figs. 6, 7 and 8, photographs are shown of pieces from the surface of the same end of one potato B. Fig. 6 shows a piece of the potato after cooking by steaming, Fig. 7 raw, untreated potato, and Fig. 8 raw, treated with nitrous acid and sodium hydroxide. A resemblance between the photographs Figs. 6 and 8 will be noted, but of course the portion of potato represented in Fig. 6 is black and in Fig. 8 red.



Fig. 1.



Fig. 2.



Fig. 3.



Fig. 4.



Fig. 5.



Fig. 6.



Fig. 7.



Fig. 8.

If the water in which potatoes are boiled is treated with nitrous acid and sodium hydroxide, coloured solutions are obtained—deep colours with water in which potatoes which blacken have been boiled and faint colours if the potatoes do not blacken.

The blackening after cooking.

It is extremely probable that this blackening is due to oxidation, but it cannot be due to enzymic oxidation, as during cooking the temperature of a potato is at about 100° for about 20 minutes. Whether the blackening after cooking is due to the oxidation of a phenol or amine has not yet been ascertained. Experiments in support of these statements as to blackening after cooking being due to oxidation are as follows.

(a) If a portion of potato B, which blackens after cooking, is placed immediately it is cooked in a gas free from oxygen and thus allowed to cool in absence of air it does not blacken.

(b) If a piece of cooked potato is placed in a bell-jar containing chloroform and water no effect such as that described above under enzymic oxidation takes place.

The effect of traces of iron on the colour of cooked potato.

It was noticed in the course of this work involving the cooking of potatoes in various ways that a black stain was occasionally obtained on a cut surface of potato when such a stain was not expected.

In one experiment some potatoes were being cooked by steaming on filter-paper in a steamer made from tinned sheet iron. The perforations in the steamer had apparently been made after the sheet iron had been tinned and the edges of the holes were rusting. It was noticed that black marks were obtained on the potatoes (after cooling in the air for some time) which coincided exactly in position with the holes in the steamer. A section of potato B showing these marks is shown in Plate III, Fig. 5.

The explanation of the black marks is probably as follows. The iron compounds from the sides of the steamer holes evidently pass through the filter-paper (as shown by rust marks on standing) and coming in contact with the potato act either catalytically in promoting oxidation, or else combine with the phenolic substances present to produce coloured substances. Most probably both actions take place. Evidence of combination of iron compounds with the phenols is obtained from the fact that somewhat violet colorations are sometimes obtained in such experiments. Ferric oxide alone does not appear to promote blackening.

It is known that it is a ferrous compound that is involved in the catalytic activity of iron in biological oxidations, and since it is iron in the process of rusting (when a ferrous compound will be present) which causes most blackening it appears that the effect of iron in promoting blackening is in part catalytic.

The effect of iron in the process of rusting on the production of blackening after cooking is well shown by steaming a peeled potato in which are inserted some newly cleaned iron nails and allowing the potato to remain exposed to the air some hours after cooking. In view of the extensive use of iron in cooking vessels the action of this metal in causing blackening after cooking must not be overlooked.

The effects of ammonia in steaming potatoes and of acid in the cooking water.

Owing to the well-known effect of ammonia in promoting atmospheric oxidation of polyhydric phenols it was found, as was expected, that potatoes steamed over water containing a little ammonium carbonate were dark in colour after cooking. The effect of the hydrogen ion concentration of the cooking water on the colour of cooked potato is to be investigated, but it has already been found that if potatoes B are cooked in water containing a little acetic acid they are usually a better colour than if cooked in tap-water.

Similarly a section of potato after soaking in dilute acid gives less colour in the nitrous acid test than a piece of the same potato which has been soaked in water only. This might point to a basic substance being concerned in the blackening process. If the acid solution and water used for soaking are subjected to the nitrous acid test a strong reaction is obtained in the acid solution, but not with the water.

Further work on this problem is in progress.

SUMMARY.

A simple test is described by means of which it is considered possible to tell whether or not potatoes will blacken after cooking.

The cause of this blackening as distinct from darkening due to enzymic oxidation is discussed, and attention is drawn to the fact that iron may have a pronounced influence on the degree of blackening observed.

My thanks are due to Miss J. Lindsay and Miss H. Tress of the Household Arts Department of this College for samples of the potatoes used in these investigations.

REFERENCES.

- Bertrand (1896). *Compt. Rend. Acad. Sci.* **122**, 1215.
Gallagher (1923). *Biochem. J.* **17**, 513.
Onslow (1919). *Biochem. J.* **13**, 1.
— (1920). *Biochem. J.* **14**, 535.

LXXXVIII. CYCLIC CHANGES IN THE GLYCOGEN CONTENT OF THE LIVER AND THE MUSCLES OF RATS AND MICE.

THEIR BEARING UPON THE SENSITIVITY OF THE ANIMALS TO INSULIN, AND THEIR INFLUENCE ON THE URINARY OUTPUT OF NITROGEN.

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(Received April 9th, 1931.)

THE physiological changes in the glycogen content of the liver have generally been considered to be the result of changes in the carbohydrate supply of the body. It was formerly regarded as established that glycogen accumulates in the liver after ingestion of food and is eliminated from the liver by fasting, especially if combined with muscular exercise. Accordingly fasting and muscular exercise have been the principal means adopted for removing the glycogen from the liver as well as from the muscular tissue. At a very early date, however, observations were made indicating that the mechanism of this function of the liver was more complex than had been supposed.

Thus it is known that glycogen reappears in the liver and the muscles of rabbits completely deprived of their glycogen by means of strychnine convulsions. If the animals are kept anaesthetised glycogen reappears in the liver within 20 to 28 hours. Pflüger [1903] suggested the possibility of a migration of glycogen from the bony and cartilaginous parts of the body. In his excellent paper on this topic Rolly [1905] indicated the possibility of glycogen formation from body proteins. On the third to fifth day of fasting, rabbits completely deprived of their glycogen by means of strychnine convulsions showed not only the reappearance of glycogen in the liver and the muscles, but also an increase in the nitrogen output through the urine. In Rolly's experiments the excess nitrogen excretion, indicating an increased protein metabolism, occurred at a time when there was no reason to suspect a pre-agonal destruction of proteins.

These early investigations tend to show that the accumulation of glycogen and its disappearance from the liver cannot be explained solely by the storage and mobilisation of the available carbohydrate material. As is contended also by Macleod in his monograph on carbohydrate metabolism, other factors,

hitherto not well-known are involved in this mechanism. Such a view is furthermore supported by recent experimental findings.

In recent times a most valuable contribution to our knowledge of glycogen metabolism has been made by Forsgren [1928]. He has observed cyclic changes in the glycogen content of the liver of rabbits, the maximum being reached at night and the minimum during the daytime. These very striking changes are to a great extent independent of nutritional conditions. Early in the day glycogen may disappear from the liver in spite of an abundant food supply, whilst at night there is an accumulation of glycogen, even if the animals are fasting. There is a corresponding periodicity in the production of bile, the latter reaching its maximum during the day, when the glycogen content of the liver is low. Forsgren obtained these results by histochemical investigations, including staining of the bile-acids and the glycogen in the liver of animals killed at different times of the day. Forsgren's results have been confirmed by chemical analysis of rabbit's liver made by two of the authors of this paper.

In order to check Forsgren's results, we conducted experiments on a rather large scale, analysing the glycogen content of the livers of a considerable number of mice and rats at different times of the day. What particularly aroused our interest in this problem was the observation that mice used in the assay of insulin consistently show a greater resistance to insulin in the evening than at noon. We thought that the diminished sensitivity of the animals to insulin in the evening might be due to an accumulation of glycogen in the liver. This possibility has been suggested by Forsgren himself [1929].

This periodicity, if established, would moreover have an important bearing on our views of carbohydrate metabolism. It is now generally assumed that in a fasting animal the glycogen content of the liver remains constant at a low level or slowly decreases. Thus Cori and Cori [1928], in their studies of the glycogen metabolism, contend that during the second day of fasting (*i.e.* between the 24th and 48th hours) a very minute amount of glycogen only can have been removed from the liver of the rat and converted into sugar. The figures found for the glycogen content of the liver were almost identical on the two different days. This constancy, however, would be quite illusory, had there been in the meantime a considerable increase of the liver glycogen followed by a decrease. The conclusion drawn in regard to the sugar metabolism during that period would consequently be erroneous. The same remark applies to many investigations of recent date on the action of insulin and adrenaline, in which the glycogen content of the body has been studied for periods ranging from 24 to 48 hours, without knowledge of these cyclic changes.

By our experiments on mice and rats we confirmed Forsgren's findings so far as concerns this periodicity in the accumulation of glycogen in the liver. We also found that it is largely independent of nutritional conditions. In spite of 40 hours' fasting a large number of the rats showed an increase in the liver-glycogen during the night. None of the rats, which had fasted more than

24 hours, if killed during the daytime, had any appreciable quantity of glycogen in the liver. The results are thus fully in agreement with the findings of Forsgren in his experiments on rabbits.

In the experiments with insulin the increase in the resistance of the mice to the hormone was found to coincide with the accumulation of glycogen in the liver. In the evening a smaller number of animals showed convulsions than at noon, and at night the dosage of insulin had to be doubled in order to induce severe intoxications in the same percentage of animals.

Having, by our first series of experiments, established the periodicity in the accumulation of glycogen in the liver, we proceeded in the subsequent series to examine also the glycogen content of the muscles. The close connection between the glycogen content of the liver and that of the muscles has been well established, especially by the studies of Cori and Cori [1929]. These authors emphatically assert that glycogen passes from the muscles to the liver and *vice versa*, the lactic acid of the blood and the blood-sugar respectively being the intermediate compounds. On this basis it seemed reasonable to presume that the glycogen content of the muscles would show a periodicity similar to that of the liver. In fact, in our three series of experiments, in which the entire body of the animals (mice) was examined for glycogen in accordance with the method of Lesser, the glycogen content of the muscles was found to be higher during the night than during the day. The difference, however, is not so marked as in the case of the liver. This seems to indicate that the glycogen formed during the night originates from the liver.

In regard to the material which gives rise to this periodical accumulation of glycogen, it may be taken for granted that an important part is played by the carbohydrates. How far the proteins also participate in this synthesis of glycogen is a problem of considerable interest. As stated above, the view that the proteins do contribute to the formation of glycogen has been emphasised by various investigators. The fact that the periodical formation of glycogen is but slowly affected by moderate fasting likewise indicates that the body proteins may participate in the process. If this is actually the case, the urinary output of nitrogen should be affected by the process. For an increased deamination of amino-acids, in connection with the accumulation of glycogen, would naturally result in an increased nitrogen output. As, so far as we know, cyclic changes in the urinary nitrogen output of experimental animals have not previously been demonstrated, we thought it desirable to investigate this question by experiments on rabbits. The results make it evident that in rabbits the nitrogen output does show a periodical variation parallel with that of the liver-glycogen.

EXPERIMENTAL.

The glycogen content of the liver was determined in seven series of experiments on mice and one series of experiments on rats. In each series 120 or 90 animals were taken. Either 10 animals were killed every 2 hours or 15

every 4 hours, beginning at 8 o'clock in the morning. All the animals belonging to one series were kept in a large cage under uniform conditions.

In two of the mouse series the animals were abundantly supplied with food and water. The stomachs of the mice on dissection were always filled with food. In four of the series each animal had fasted 10 hours before being killed. Previously to the latter experiments 10 animals had been placed every 2 hours in smaller cages, supplied with water only. In one of the mouse series the food had been removed 10 hours before the beginning of the experiment, so that those last killed had fasted 34 hours. The rats had fasted 24 hours before the beginning of the experiment.

The animals after weighing were killed by decapitation. The liver was rapidly removed, weighed, cut in pieces and placed in a centrifuge tube together with 1 cc. of 60 % potassium hydroxide. The tube was immersed in a boiling water-bath. The liver of a mouse is sufficiently large for a single analysis. Of the rat's liver about 1 g. was taken.

Where the glycogen content of the whole animal was determined in accordance with the method of Lesser, the mouse, after the removal of the liver, was immediately cut up with a pair of scissors, and placed in a large centrifuge tube, containing 20 cc. of hot potassium hydroxide solution. The alkaline solution, on heating in a water-bath, quickly penetrated the material. The heating was continued for 3 hours; residual bony material was removed before the precipitation with alcohol.

After the hydrolysis 1 cc. of the potassium hydroxide solution was treated with 2 cc. of water and 8 cc. of 95 % alcohol. The precipitate was washed in the centrifuge tube 3 times with 66 % alcohol, 3 times with 95 % and once with absolute alcohol. After washing with ether and drying, it was hydrolysed by heating for 3 hours in a water-bath with 5 % hydrochloric acid. The sugar determination was made according to the method of Bertrand.

By the method used 2-5 mg. glycogen were recovered with a loss not exceeding 3 %. In comparing these results with the figures found on analysis of 25 g. of the liver according to the macro-method, the differences between the figures were found to be less than 5 %.

In the series where insulin was injected into mice, the animals were taken from a stock of several hundreds kept in a large box. In two of the series (Figs. 3 and 4) 50 animals were taken out of the box 10 hours before they were injected with insulin. They were supplied with water only. In one series (Fig. 6) all of them had been deprived of food at 10 p.m. on the preceding day. In each of the experiments recorded in the figures 50 animals were injected with an amount of insulin, known to cause convulsions in 50 % of cases within 2 hours. The amount of insulin necessary was 0.0062-0.0075 units per 20 g. body weight, which was dissolved in 0.5 cc. of saline and injected subcutaneously. The temperature was kept constant at 38°.

The experiments concerning the urinary nitrogen output were performed on female rabbits. The animals were kept in small cages which did not permit

free movements. The cages were provided with bottoms of strong wire netting and were placed in aluminium bowls for the collection of urine. The urine was collected twice daily at 8.30 a.m. and 8.30 p.m. On each occasion the bladder was rinsed with water through a metal catheter. In order that the animals might be supplied with water 60 to 80 cc. of saline was at the same time injected subcutaneously. The urinary excretion was thus followed during a period of 6 days, throughout which the animals were kept fasting. The temperature and the body weight were recorded every other day.

RESULTS AND CONCLUSIONS.

Fig. 1 shows the glycogen content of the liver of animals (mice) abundantly supplied with food. The glycogen content was much higher during the night

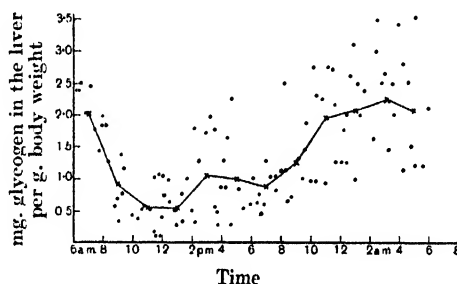


Fig. 1. Glycogen content of the liver of mice abundantly supplied with food.

than during the day. The average figures for 10 animals were about 100 % higher in the night than in the day. In Fig. 1 (Table I, A) the figures for the liver-glycogen of the 50 animals killed between 10 a.m. and 8 p.m. were

Table I. *Cyclic variations in glycogen of mice and rats under different conditions.*

(Figures represent μ g. glycogen/g. body weight (and average deviation from mean) determined in groups of 10 animals.)

Time ...	8 a.m.	10 a.m.	12 noon	2 p.m.	4 p.m.	6 p.m.
A. Mouse-liver; animals well-fed	0.93 (± 0.177)	0.56 (± 0.139)	0.62 (± 0.077)	1.10 (± 0.213)	1.02 (± 0.207)	0.86 (± 0.093)
B. Rat-liver; animals fasted 24 hrs. before and during experiment	0.028 (± 0.00225)	0.034 (± 0.00438)	0.033 (± 0.0051)	0.031 (± 0.0062)	0.027 (± 0.0033)	0.115 (± 0.0348)
C. Mouse-liver; animals fasted 10 hrs.	2.22 (± 0.203)	1.67 (± 0.248)	1.20 (± 0.244)	0.54 (± 0.161)	—	0.38 (± 0.049)
D. Mice as under C. Body exclusive of liver	0.57 (± 0.082)	—	—	0.49 (± 0.078)	—	—
Time ...	8 p.m.	10 p.m.	12 midnight	2 a.m.	4 a.m.	6 a.m.
A. Mouse-liver; animals well-fed	1.28 (± 0.184)	1.94 (± 0.243)	2.06 (± 0.216)	2.51 (± 0.243)	2.04 (± 0.264)	2.08 (± 0.125)
B. Rat-liver; animals fasted 24 hrs. before and during experiment	0.036 (± 0.0047)	0.061 (± 0.0195)	0.076 (± 0.0261)	0.139 (± 0.0272)	0.104 (± 0.0329)	0.083 (± 0.0203)
C. Mouse-liver; animals fasted 10 hrs.	0.34 (± 0.062)	—	1.03 (± 0.158)	—	0.81 (± 0.094)	1.57 (± 0.099)
D. Mice as under C. Body exclusive of liver	0.25 (± 0.0)	—	0.47 (± 0.083)	—	0.62 (± 0.0026)	—

0.83 ± 0.0679 mg./g. body weight and for the 59 killed between 8 p.m. and 8 a.m. 1.97 ± 0.0986 . Thus under normal conditions glycogen accumulates in the liver of the mouse during the night.

Similar changes occur in the liver even if the animals are deprived of food. They could be demonstrated in mice after 10 hours' fasting and in rats which had been kept without food for 40 hours. Fig. 2 shows the results

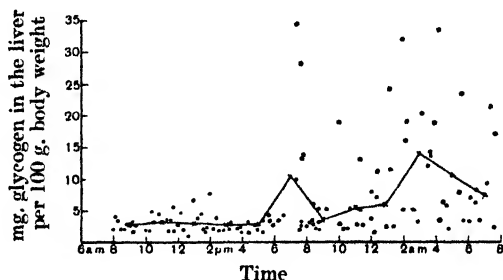


Fig. 2. Glycogen content of the liver of rats, which had fasted 24 hours before the experiment and were kept fasting.

obtained with rats which had fasted 24 to 48 hours. In the daytime the liver contained about 3 mg. glycogen per 100 g. body weight, whereas the figures during the night were 8–10 mg. The 70 animals killed between 10 a.m. and

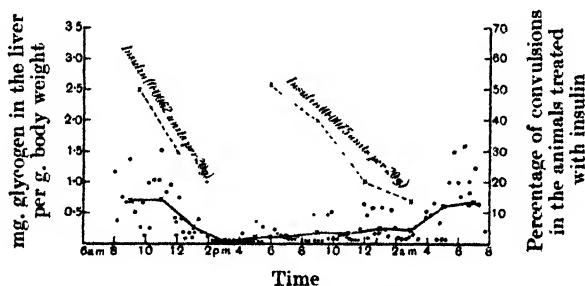


Fig. 3. Glycogen content of the liver of mice, which had fasted 10 hours before analysis.

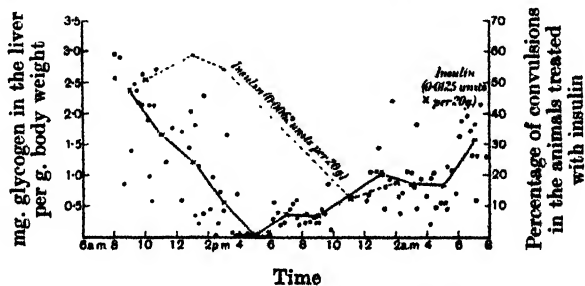


Fig. 4. Glycogen content of the liver of mice, which had fasted 10 hours before analysis.

12 midnight showed an average of 4.8 ± 0.681 , the 50 killed between 12 midnight and 10 a.m. 8.6 ± 1.17 . The figures are found in Table I, B. Figs. 3 and 4

give the glycogen content of the liver of mice, which had fasted 10 hours before killing. The same periodicity is shown in these figures, especially in Fig. 4. Here the average figure for the glycogen content of the liver of the 40 animals killed between 2 p.m. and 12 midnight was 0.42 ± 0.060 mg. per g. bodyweight, whereas the figure for the 70 animals killed between 8 a.m. and 2 p.m. and between 12 midnight and 8 a.m. was 1.32 ± 0.0867 . The figures are given in Table I, C. The glycogen content of the livers of the 60 animals (see Fig. 3) killed between noon and midnight was 0.16 ± 0.078 and of the 57 killed between midnight and noon 0.53 ± 0.057 mg. per g. body weight.

The urinary nitrogen output was followed in three series with 10 rabbits in each. The animals were kept fasting for a period of 6 days, during which time the urine was collected quantitatively and analysed for nitrogen. Throughout all the series it could be established that the urinary nitrogen output was larger during the night than during the daytime. Owing to several circumstances the first two series yielded incomplete results. We therefore report only the third series (see Table II). In the second series the nitrogen output during

Table II. *Urinary nitrogen of rabbits excreted during periods of 12 hours.*

Rabbit	Time	mg. nitrogen.					
		Day of fasting					
		1	2	3	4	5	6
1	8.30 p.m.-8.30 a.m.	779 + 19	903	697 + 17	721	695 + 15	562
	8.30 a.m.-8.30 p.m.	655	780 + 16	591 + 17	688 + 5	598 + 15	481 + 17
2	8.30 p.m.-8.30 a.m.	680	861	732	592 + 22	457	588
	8.30 a.m.-8.30 p.m.	868 - 22	713 + 21	532 + 37	486 + 22	512 - 11	491 + 20
3	8.30 p.m.-8.30 a.m.	778 + 2	865	773	628 + 8	685	750
	8.30 a.m.-8.30 p.m.	763	792 + 9	632 + 22	580 + 8	717 - 4	669 + 12
4	8.30 p.m.-8.30 a.m.	835 + 5	935	832	758 + 23	746	554
	8.30 a.m.-8.30 p.m.	798	819 + 14	712 + 17	616 + 23	622 + 20	487 + 14
5	8.30 p.m.-8.30 a.m.	1005 + 29	1100	1043	632	578	623
	8.30 a.m.-8.30 p.m.	780	895 + 23	782 + 33	552 + 14	476 + 21	438 + 42
6	8.30 p.m.-8.30 a.m.	875	1010	.	812	654	600
	8.30 a.m.-8.30 p.m.	768 + 14	840 + 20	875	676 - 21	578 + 13	495 + 21
7	8.30 p.m.-8.30 a.m.	698	817	820	674	572	485
	8.30 a.m.-8.30 p.m.	.	691 + 18	669 + 23	596 + 14	509 + 12	466 + 4
8	8.30 p.m.-8.30 a.m.	.	928	882	660	641	634
	8.30 a.m.-8.30 p.m.	673	823 + 13	709 + 24	618 + 7	588 + 9	565 + 12
9	8.30 p.m.-8.30 a.m.	952	853	868	598	529	546
	8.30 a.m.-8.30 p.m.	626 + 52	.	642 + 35	542 + 10	512 + 3	436 + 25
10	8.30 p.m.-8.30 a.m.	840	753	855	.	718	696
	8.30 a.m.-8.30 p.m.	576 + 46	728 + 3	714 + 20	575	540 + 33	640 + 9

the night was, on an average, 16 % larger than in the daytime for the first 3 days and 6.6 % larger during the last 3 days of the fasting. In the third series we succeeded in collecting the urine on each occasion except 6 out of a total of 120. The table shows a constantly increased output of nitrogen during the night, which persists in spite of the continued fasting.

Only on 3 occasions out of 55, on which the morning and the evening figures were recorded, did we observe a lower output of nitrogen in the

morning than in the evening. To judge by the evening figures, the low output on one of these occasions (rabbit No. 2, first day) was certainly due to a defective evacuation of the bladder in the morning, and on another occasion (the same rabbit on the 5th day) it was probably due to the same reason.

In the table the differences between the morning and the evening figures are given as percentages of the evening values. If we take the average for the positive figures, the increased output during the night amounts to 21.2 % for the first 3 days and to 15.8 % for the last 3.

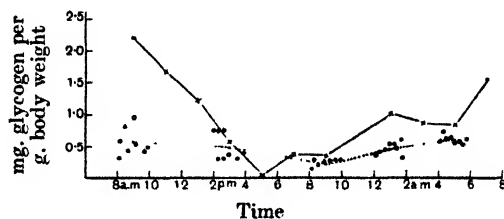


Fig. 5. Glycogen content of the muscular tissue ($\times \cdots \cdots \times$) of mice, which had fasted 10 hours before analysis, as compared with the glycogen content of the liver of the animals (\times — \times).

In the series recorded in the table the rabbits showed no rise of temperature and a moderate decrease of weight, so that the results were not distorted by any complications.

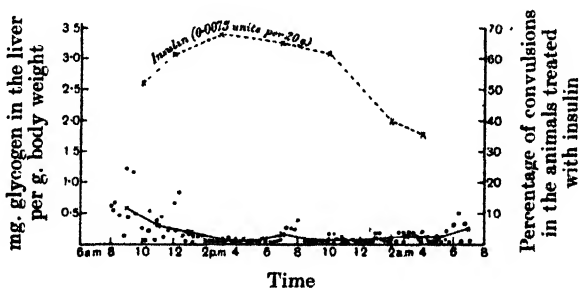


Fig. 6. Glycogen content of the liver of mice, which had fasted 10 hours before the experiment and were kept fasting.

It may be presumed that this increased nitrogen output during the night is genetically connected with the formation of glycogen. The cyclic accumulation of glycogen in the liver may be attributed partly to a conversion of proteins into glycogen. If we suppose that a rabbit excretes 1.5 g. nitrogen in 24 hours, the increased output during the night would be about 0.1–0.2 g., corresponding to approximately 0.4–1.0 g. glycogen. Thus in a rabbit's liver weighing 60–70 g. up to 1.5 % of glycogen, or possibly somewhat more, could be formed solely from the excess of amino-acids deaminised during the night. It seems reasonable to assume that the body proteins participate in this process, seeing that it is only slightly affected by fasting.

The excess output of nitrogen in these experiments, which is about 10 %

of the total amount of nitrogen eliminated, must represent the minimum amount of proteins thus converted into glycogen. It may, however, be presumed that the proteins metabolised are transformed into glycogen to a much larger extent than can be traced in this way.

SUMMARY.

There are cyclic changes in the glycogen content of the liver of rabbits, rats and mice, which are to a large extent independent of the intake of food. Glycogen accumulates in the liver during the night and disappears again to some extent during the next morning. The same occurs even in fasting animals.

Similar periodical changes occur, though to a minor extent, in the muscles also.

As might have been expected from the observations recorded above, mice show an increased resistance to insulin in the afternoon and particularly during the night.

In rabbits, where these periodical changes are particularly striking, the urinary nitrogen output is increased during the night. In fasting rabbits the excess of nitrogen thus eliminated is about 20 % of the amount excreted during the day. This indicates that an increased deamination of amino-acids occurs during the night and that the formation of glycogen takes place partly at the expense of the body proteins.

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REFERENCES.

- Cori and Cori (1928). *J. Biol. Chem.* **79**, 309.
- - - (1929). *J. Biol. Chem.* **81**, 389.
Forsgren (1928). *Z. Zellforsch. mikr. Anat.* **6**, 647.
- - - (1929). *Acta Med. Scand.* **70**, 139.
Pflüger (1903). *Pflüger's Arch.* **96**, 1.
Rolly (1905). *Deutsch. Arch. klin. Med.* **83**, 107.

LXXXIX. METABOLISM STUDIES IN TETANY.

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A DISTURBANCE in chlorine metabolism has long been associated as an etiological factor in the production of certain forms of tetany. MacCallum and his co-workers [1920] attributed the onset of tetany in cases of pyloric obstruction to the loss of the gastric hydrochloric acid by the vomitus with the consequent production of an alkalosis. Grant [1922] found in one of six cases of adult tetany a marked reduction in the blood-chlorine. Iversen and Hansborg [1922] showed that after thyroidectomy sodium chloride was retained in the tissues to an abnormal extent although the amount of chlorine in the blood was reduced. Parhon, Ballif and Derevici [1926] also reported a fall in the serum-chlorine. Rockemann [1923] found that tetany following injection of sodium dihydrogen phosphate was accompanied by decreased excretion of urinary chloride.

The present investigation was undertaken primarily to study the changes in chlorine metabolism during conditions of tetany not associated with vomiting. Much work, however, has already been done indicating that in many forms of tetany there is a profound disturbance in calcium metabolism. Numerous experimental studies by Berkeley and Beebe [1909], Cooke [1911] and others showed that after parathyroidectomy there occurs a definite increase in the output of nitrogen, while Burns [1917] recorded a similar finding following guanidine injection. The first section of this paper accordingly deals with the effect of tetany on chlorine metabolism, while succeeding parts describe the alterations in nitrogen and mineral metabolism.

Methods.

Tetany was induced in cats by removal of the parathyroids and in rabbits by injection of guanidine. The parathyroids were first removed under chloroform anaesthesia; this usually produced a condition of latent tetany indicated by sluggishness and the other characteristic manifestations. A second operation of complete thyroidectomy led to the development of active tetany. The effect of guanidine was investigated by the subcutaneous injection of a 6 % solution of guanidine carbonate. 5 cc. were used in each case except rabbit 2

where the dose was 7 cc. An interval of 6 days elapsed between succeeding injections. The food was weighed and aliquot portions of each article of diet were analysed. The unused residues of the diet were kept, thoroughly mixed and analysed. Accordingly the intake during each period of the experiment was accurately known. The rabbits were fed on a mixture of equal parts of oats, bran and linseed, while the cats received oatmeal porridge and milk. Urine and faeces were collected separately. The urine was analysed daily while the faeces for each period were collected, dried on a steam-bath and analysed. The methods of analysis employed were: chloride [Van Slyke, 1923]; calcium [McCrudden, 1911]; phosphorus, Neumann, total nitrogen, Kjeldahl; purines, modified Camerer method [Cathcart *et al.*, 1925], creatine and creatinine, Folin; ammonia, Folin aeration method; amino-acids, Sørensen.

CHLORINE.

Results.

A. *Urine* (Table I). It is clear that the urinary output of chlorine was diminished after removal of parathyroids or injection of guanidine much more than can be accounted for by the decreased intake. Accordingly there

Table I. *Showing intake, output and retention of chlorine in mg. daily.*

Cat	Period	Intake	Output	Re- tention	% re- tention
1	1. Normal	790.6	672.5	118.1	14.9
	2. Latent tetany	718.7	496.0	222.7	30.9
	3. Recovery	830.4	529.4	301.0	36.3
	4. Active tetany	194.2	Nil	194.2	100.0
	5. Recovery	706.0	1267.0	Negative	Negative
2	1. Normal	919.0	906.7	12.3	1.3
	2. Latent tetany	1000.0	642.0	358.0	35.8
3	1. Normal	707.4	583.6	123.8	17.5
	2. Latent tetany	528.0	157.4	370.6	70.1
	3. Active tetany	676.3	148.4	527.9	78.0
	4. Recovery	707.4	564.0	143.4	20.2
4	1. Normal	400.7	379.6	21.1	5.2
	2. Latent tetany	400.4	260.4	140.0	34.9
5	1. Normal	431.4	372.7	58.7	13.6
	2. Latent tetany	430.0	287.0	143.0	33.2
Rabbit					
1	1. Normal—7 days	30.6	30.1	0.5	1.63
	2. Guanidine—5 days following 1st injection	18.2	8.6	9.6	52.7
	3. Guanidine—5 days following 2nd injection	8.55	3.0	5.55	64.9
	4. Guanidine—10 days following 3rd injection	7.1	1.4	5.7	80.2
2	1. Normal—7 days	28.4	31.3	12.9	—
	2. Guanidine—5 days after 1st injection ...	7.1	4.04	3.06	43.1
3	1. Normal—7 days	42.6	41.2	1.4	3.3
	2. Guanidine—5 days following 1st injection	16.05	13.33	2.72	16.9
	3. Guanidine—5 days following 2nd injection	10.2	4.76	5.44	53.3
	4. Guanidine—10 days following 3rd injection	14.2	5.1	9.1	64.1
4	1. Normal—7 days	44.4	42.9	1.5	3.4
	2. Guanidine—5 days following 1st injection	8.66	4.54	4.12	47.5
	3. Guanidine—5 days following 2nd injection	7.1	2.44	4.66	65.6
	4. Guanidine—10 days following 3rd injection	8.6	2.40	6.20	72.1

was actually an increased retention of chlorine during both latent and active tetany periods although the intake was in most cases much less than that of the normal. If sufficient chlorine had been ingested the retention was more marked in the active stage.

B. *Blood* (Table II). The immediate effect of an injection of guanidine on the total chlorine content of the blood was with one exception (rabbit 11) a reduction of 8 to 18 milli-equiv. per litre. This reduction was followed within

Table II. *Showing total and volatile chlorine of blood in normal condition and active and latent tetany (m.-eq. per litre).*

Period	Cat 1		Cat 3		Cat 5		Cat 6		Cat 7		Cat 8	
	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl
Normal	102.6	14.6	101.8	11.4	96.2	10.4	98.6	11.8	104.7	12.2	98.0	10.0
	—	—	100.4	10.4	97.1	11.2	100.1	11.8	106.0	12.0	100.2	10.4
	—	—	—	—	—	—	—	—	106.2	11.7	100.3	10.5
Latent tetany	—	—	—	—	—	—	91.2	12.9	93.1	16.3	92.5	12.1
	122.1	19.8	—	—	109.5	20.4	110.5	18.1	113.2	17.4	112.6	14.6
	—	—	—	—	108.7	18.3	112.8	20.6	115.9	17.3	115.9	19.8
	—	—	—	—	110.5	12.3	112.8	20.3	113.8	20.0	115.4	19.2
Active tetany	—	—	—	—	110.0	0.2	—	—	102.8	0.0	107.5	1.0
Recovery	105.8	12.6	103.0	11.6	—	—	—	—	—	—	—	—

Period	Rabbit 1		Rabbit 2		Rabbit 3		Rabbit 4		Rabbit 5		Rabbit 6	
	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl
Normal	80.2	8.6	74.6	13.2	85.0	8.6	77.6	11.2	82.5	10.0	67.3	11.2
	80.0	10.2	75.1	12.6	84.8	10.4	78.9	10.8	80.6	10.0	67.4	11.5
1st day after 1st injection	70.3	0.0	75.5	0.7	76.8	0.8	62.5	3.6	70.3	2.1	50.8	3.5
4th day after 1st injection	81.8	17.4	80.4	15.3	88.2	19.3	80.9	20.0	80.6	17.8	75.5	16.3
1st day after 2nd injection	72.9	-0.1	—	—	75.8	2.3	70.7	2.2	68.9	4.5	58.8	5.1
4th day after 2nd injection	86.6	17.7	—	—	80.4	19.6	86.5	18.6	78.8	18.4	75.9	16.1
1st day after 3rd injection	78.8	-0.2	—	—	75.9	1.1	80.4	4.0	75.5	0.3	70.2	0.3
8th day after 3rd injection	73.0	12.6	—	—	73.0	0.6	78.1	13.3	67.7	7.2	75.5	10.7

3 to 4 days by a return to a normal or slightly super-normal figure except in the period following the third injection. In two cases after the third injection a return to normal was recorded while in the other three the total chlorine was still further reduced. The volatile chlorine of the blood almost vanished immediately after administration of guanidine, only to return 4 days after the injection to a value exceeding the normal figure.

Similar results were noted following the removal of parathyroids. The onset of latent tetany was accompanied by a decrease of the total chlorine of the blood with a rise in the volatile portion. As the state of latent tetany was prolonged the total chlorine rose to a value 10-15 % in excess of the normal, while the volatile chlorine became almost twice as great as in health. When active tetany was induced by a complete thyreo-parathyroidectomy the total chlorine still remained above normal in two of the three cases, while

the volatile form almost completely disappeared. In two cats in which as the result of treatment with saline there were no evident symptoms the blood-chlorine values were normal.

C. *Tissues* (Table III). Analysis of the tissues showed that in both the latent and active stages of tetany due either to parathyroidectomy or guanidine poisoning there occurred a marked increase of the total Cl of muscle, heart

Table III. *Showing total and volatile chlorine of tissues in normal condition and active and latent tetany (m.-eq. per litre).*

Cat	Condition	Muscle		Heart		Liver		Kidney		Lung	
		Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl	Total Cl	Vol. Cl
A	Normal	15.0	1.8	32.1	6.1	27.2	3.4	41.0	7.0	61.1	11.1
B	"	13.6	4.1	—	—	26.3	7.8	40.0	4.4	—	—
C	"	14.2	2.2	26.6	6.2	31.5	2.3	35.0	5.0	62.5	11.7
	Average	14.3	2.7	29.4	6.2	28.3	4.5	38.7	5.5	61.8	11.4
6	Latent tetany	51.0	28.2	67.0	36.4	34.5	19.7	51.2	9.2	57.7	22.0
2	Active tetany	63.6	2.1	77.8	0.0	62.2	0.1	56.3	0.0	42.0	0.2
5	"	63.8	3.1	86.0	0.7	66.0	0.0	63.3	0.0	51.2	0.0
7	"	54.0	0.2	85.0	0.1	40.0	1.7	64.0	0.0	57.2	1.1
8	"	76.5	0.7	48.4	0.4	60.4	0.2	40.8	0.8	55.9	2.9
	Average	65.0	1.5	74.3	0.3	57.2	0.5	56.1	0.2	51.6	1.1
1	Recovered	46.0	5.6	50.5	20.4	28.2	5.8	50.0	5.0	54.0	4.9
3	"	23.0	3.0	40.0	16.0	30.0	5.2	53.0	4.0	51.0	21.4
	Average	34.5	4.3	45.3	18.2	29.1	5.5	51.5	4.5	52.5	13.2
Rabbit											
7	Normal	7.3	3.7	34.2	9.9	27.6	3.5	—	—	48.8	16.2
8	"	11.0	3.9	36.0	9.6	22.0	2.0	48.6	14.1	50.0	15.9
9	"	15.6	4.7	34.8	8.5	23.0	5.1	53.8	19.6	49.2	13.0
	Average	11.3	4.1	35.0	9.3	24.2	3.5	50.7	16.9	49.3	15.0
3	Latent tetany	35.0	25.2	55.0	12.5	39.5	24.3	37.8	10.4	43.5	14.7
4	"	31.0	19.9	52.0	7.0	41.4	14.9	44.0	9.9	41.4	11.6
5	"	41.0	32.8	55.4	12.2	42.4	24.1	43.2	25.6	47.7	17.7
	Average	35.7	26.0	54.1	10.6	41.1	21.1	41.7	15.3	44.2	14.7
1	Active tetany	46.0	0.2	54.6	0.6	40.1	1.5	23.7	0.8	46.3	0.3
2	"	42.4	0.5	56.4	0.4	38.9	0.1	29.6	1.0	47.2	0.4
6	"	40.0	2.8	55.7	1.9	37.8	1.8	28.5	1.4	41.2	1.2
	Average	42.8	1.2	55.6	1.0	38.9	1.1	27.3	1.1	44.9	0.6

and liver. Following injection of guanidine the total Cl of kidney and lung was reduced, while after removal of the parathyroids the Cl was increased in the kidney but reduced in the lung. During the latent tetany stage the volatile Cl was increased markedly in muscle and liver and slightly in kidney in both series of experiments. In heart and lung the volatile portion was unchanged in the guanidine-treated group but definitely increased in the parathyroidectomy series. During active tetany in both groups there was almost complete disappearance of volatile Cl. Two cats, which were killed during a period of freedom from all manifest signs of tetany, showed a moderate increase in the total Cl content of heart and skeletal muscle but otherwise practically normal values.

Discussion.

Guanidine. From these results it is permissible to conclude that guanidine poisoning creates a demand for chlorine in the tissues. The early fall in the total chlorine content of the blood coupled with the diminution in the urinary excretion is clearly a consequence of the withdrawal of chlorine to the tissues. The subsequent rise of the blood-chlorine is in all probability the result of gradual repletion of the blood from the chlorine ingested.

It has already been shown that chlorine exists in part as a volatile organic compound which has the power of forming a complex with substances containing the amino-group [Morris and Morris, 1930]. When so combined the power of volatilisation at 100° is lost. The amount of non-volatile amino-chlorine complex depends on the proportion of volatile organic chlorine to amino-groups, closely following the adsorption formula. The changes in the content of volatile chlorine of blood and tissues are strongly suggestive of the formation of amino-compounds in the tissues. Within 24 hours of the injection of guanidine the volatile chlorine of the blood has completely disappeared while the non-volatile fraction remains practically unaltered in amount. As there is no excessive excretion of chlorine it must be concluded that the volatile chlorine has gone to the tissues. Four days after the injection the volatile chlorine in the blood has increased above its normal value. Meanwhile in order to satisfy the extra demands there is an excessive retention of the chlorine ingested. The results of tissue analysis during the latent tetany stage conform with this view. It is seen that during that period there is a great increase in the total chlorine, affecting in muscle and liver only the volatile form. Renal tissue, however, contains rather less chlorine in this period, the result probably of less chlorine having been carried to the kidney for excretion. It is possible that the increase of volatile chlorine is due to the presence of guanidine itself. If that were so it would be reasonable to expect either that it should be more or less uniformly distributed over the tissues or that excess should be found only in the liver, the chief seat of deamination. It is significant, however, that skeletal muscle, one of the chief centres of metabolic activity, shows equally with liver a marked increase in the amount of volatile chlorine.

In the stage of active tetany the striking feature of the results of tissue analysis is the great diminution in volatile chlorine, which must be due to the presence of excessive amount of amino-compound. The total chlorine of all the tissues except kidney remains high as in the latent tetany stage.

Parathyroidectomy. The results show that chlorine metabolism was affected in almost the same manner as in guanidine poisoning. In several instances intraperitoneal injection of sodium chloride led to a rapid disappearance of the symptoms. This was shown by Paton and Findlay [1917] who attributed the beneficial action of the saline to a dilution of the poison. It seems from the present study more correct to refer the effect of administered

chloride to a neutralisation of the protein breakdown product by the formation of an amino-chlorine complex.

It appears justifiable, therefore, to conclude that the production of tetany by guanidine poisoning or by parathyroidectomy is the result of excessive proteolysis leading to the liberation of a large amount of amino-nitrogen which unites with the volatile chlorine. As long as the ratio of volatile chlorine to amino-nitrogen is maintained above a certain value manifest symptoms of tetany do not appear. When the liberation of amino-nitrogen proceeds apace and reduces this ratio the volatile chlorine disappears owing to the formation of a non-volatile complex, and active tetany ensues. Should the ratio again be raised by the parenteral administration of chlorine, owing to the rapid formation of volatile chlorine the symptoms again become latent. It is well known that the feeding of meat hastens the onset of tetany, a result to be expected in view of the increased amount of circulating amino-nitrogen. This view would explain the discordant results recorded of guanidine poisoning. Obviously the production of manifest tetany would depend on two factors, (a) the extent of protein breakdown and (b) the available supply of chlorine. The greater the supply of chlorine the larger would be the dose of guanidine necessary to produce symptoms. Further, the guanidine having itself an amino-group might conceivably be detoxicated before it could act on the tissues, provided there was available an abundance of chlorine.

Still further support is lent to our hypothesis by a consideration of gastric tetany. In this form there is a great loss of chlorine by the vomitus leading to a great deficiency in the tissues, which coincides with normal or slightly excessive liberation of amino-nitrogen. Further, it is of interest to state that in the idiopathic tetany of children there also occurs a marked retention of chlorine [Morris and MacRae, 1931].

NITROGEN.

The results noted in Table IV indicate that, following parathyroidectomy, in spite of decreased intake the urinary output of nitrogen was markedly increased so that the retention was greatly diminished, there being in four instances a net loss. The faecal nitrogen content varied but little. This is in accord with the numerous experimental studies previously recorded by Berkeley and Beebe [1909] and others for tetania parathyreopriva and by Burns [1917] for guanidine tetany. The partition of nitrogen in the urine is of importance in indicating the source of the excess nitrogen. The results heretofore recorded in the literature are conflicting. Berkeley and Beebe [1909] found an increase in the ammonia of the blood and urine and because of this and of the effectiveness of calcium therapy in parathyroid tetany and ammonia intoxication concluded that the excess of ammonia was the causal agent in the production of tetany. Falta and Kahn [1912] and Underhill *et al.* [1922] also found an increase in ammonia whereas Carlson and Jacobson [1911], Greenwald [1913], Albertoni [1914] could only show a slight rise in the ammonia

Table IV. *Showing intake, output and retention of nitrogen in g. daily.*

Cat	Period	Intake	Output			Reten- tion	NH ₃	Total* crea- tinine	Amino- acid- N	Purine*- N
			Urine	Faeces	Total					
1	Normal	3.5200	1.2400	1.6209	2.8600	0.6592	—	167.40	101.52	3.478
	Latent	3.2700	1.9725	1.7100	3.6825	0.4125	—	50.20	359.56	31.500
	Active	0.7205	1.5170	0.0000	1.5170	0.7965	—	70.70	203.60	30.080
	Recovery	2.9100	0.8824	0.6911	1.5174	1.3926	—	124.40	172.02	3.500
2	Normal	5.7370	1.7130	3.5409	5.2530	0.4840	—	234.36	113.40	6.456
	Latent	5.7000	2.8180	3.7610	6.5790	0.8790	—	66.75	344.25	20.410
3	Normal	3.9000	0.6886	0.2781	0.9667	2.9333	0.1294	137.00	63.95	2.514
	Latent	1.3730	0.8770	0.0870	0.9140	0.4590	0.0346	39.36	301.25	37.829
	Active	0.7080	2.1443	0.0000	2.1443	1.4363	0.0239	53.96	302.00	105.148
	Recovery	3.9000	0.6540	0.2581	0.9121	2.9879	0.0681	110.92	62.83	9.201
4	Normal	3.5300	0.5960	0.2820	0.8780	2.6520	0.1074	220.32	43.38	1.538
	Latent	2.2800	0.8430	0.1280	0.9710	1.3090	0.0507	135.66	47.54	41.025
5	Normal	2.6140	0.7306	0.0900	0.8206	1.7934	0.1279	243.00	70.65	1.032
	Latent	2.0500	0.9512	0.0821	1.0333	1.0167	0.0515	157.00	60.13	44.600
Rabbit										
1	Normal	1.4301	0.7767	0.3240	1.1007	0.3294	0.0296	24.50	61.30	9.87
	1st injection	0.7322	0.3986	0.3329	0.7315	0.0007	0.0165	25.10	38.60	11.80
	2nd "	0.3000	0.2310	0.2225	0.4535	-0.1535	0.0014	4.90	82.10	31.72
	3rd "	0.2860	0.3137	0.2005	0.5142	-0.2282	0.0004	—	131.40	33.87
2	Normal	1.1072	0.5997	0.2490	0.8487	0.2585	0.0370	24.50	22.00	12.04
	1st injection	0.2860	0.2460	0.1380	0.3840	-0.0980	0.0086	25.00	53.40	12.01
3	Normal	1.7875	0.8857	0.6000	1.4857	0.3018	0.0096	17.46	27.75	12.90
	1st injection	0.6464	0.3852	0.2082	0.5934	0.0530	0.0076	18.05	17.58	10.20
	2nd "	0.4118	0.3148	0.1071	0.4219	-0.0101	0.0018	5.18	41.84	30.70
	3rd "	0.5720	0.5839	0.1109	0.6948	-0.1228	0.0008	—	174.40	25.40
4	Normal	1.8000	0.8420	0.5850	1.4270	0.3730	0.0113	13.53	20.00	9.87
	1st injection	0.3489	0.1870	0.1800	0.3670	-0.0181	0.0123	13.00	16.08	10.00
	2nd "	0.3000	0.3201	0.1075	0.4276	-0.1276	0.0088	5.80	81.00	24.27
	3rd "	0.3000	0.4333	0.1069	0.5402	-0.2402	0.0016	—	152.80	22.89

* The figures for creatinine, amino-acid-N and purine-N are in mg. daily.

excreted in the urine and no excess in the blood. Carlson and Jacobson [1911] also showed that there is a difference in the therapeutic actions of calcium salts in ammonia poisoning and tetany. Wilson, Stearns and Thurlow [1915] found a lowering of the ammonia coefficient during latent, but a rise during active tetany. MacCallum and Voegtlin [1911] on the other hand obtained a definite increase immediately after parathyroidectomy. Cooke [1910] and Palladin and Grilliches [1924] reported in tetania parathyreopriva an increase in the total creatinine output whereas Burns [1917] obtained the opposite result after parathyroidectomy but a slight increase in guanidine tetany. Esau and Stoland [1930] concluded that the initial effect of parathyroidectomy is on tissue metabolism, the substance chiefly affected being phosphagen. Justschenko [1913] found an increase in the output of amino-acids, while Falta and Kahn's [1912] results indicated no change in the excretion of amino-acids but a rise in the peptide-nitrogen of the urine. Burns [1917] reported a slight rise in the output of amino-acids after one injection of guanidine.

Our results demonstrate the following points.

Total creatinine. After removal of the parathyroids there was a definite decrease in the amount of total creatinine during latent and active tetany with a tendency to return to normal in the recovery stage. After the first

injection of guanidine, however, the output was unchanged or slightly increased but following the later injections it was markedly diminished. After the third injection creatinine could not be detected in the urine.

Ammonia. The amount excreted was diminished, the decrease becoming more marked as the tetany became more manifest.

Amino-acids. After parathyroidectomy the amino-acid content of the urine was markedly increased in all the tetany periods except two (in one it was practically unchanged and in the other somewhat reduced). Following the first injection of guanidine there occurred except in rabbit 2 a decrease in the amount of amino-acid excreted. The later injections of guanidine led to a marked increase in the output.

Purine-nitrogen. In all cases the output of purine-nitrogen was definitely increased.

From these results it seems fair to conclude that an immediate and important effect of parathyroidectomy and guanidine poisoning is an excessive tissue catabolism. It also seems that the effect on nitrogen metabolism is roughly parallel with the degree of tetany produced. This is more apparent in the guanidine series where it is evident that the effect of guanidine is dependent on the dosage. The second and third injections produced more marked effects.

MINERALS.

It is clear that comparison between the retentions of calcium and phosphorus in the various periods is difficult owing to the difference in intakes. Accordingly we have estimated the retention as a percentage of the intake. In calculating the difference in the amount of calcium or phosphorus excreted we have calculated the expected excretion of the normal period on the same calcium or phosphorus intake as that of the tetany period. This admittedly is not satisfactory, but the fact that we obtain such close correlation between the excess calcium calculated on this basis and the excess amino-acids seems to afford some justification for this method of computation. The only other course is to withhold food entirely during all the periods. Such a procedure is open to the very serious objection that ketosis with all its concomitant metabolic effects complicates the picture.

Calcium.

Our results (Table V) may be summarised as follows.

After removal of the parathyroids the urinary output of calcium was increased in the stage of latent tetany and still more in the active period, the faecal output was decreased but not markedly so and the retention of calcium calculated as a percentage of the intake was decreased.

After the first injection of guanidine there occurred a decrease of urinary calcium. The second and still more the third administration of guanidine led to a rise in the amount of calcium in the urine. The percentage retention was

Table V. *Showing intake, output and retention of calcium and phosphorus in g. daily.*

Cat	Period	Calcium						Phosphorus					
		Intake	Output			Retention		Intake	Output			Retention	
			Urine	Faeces	Total	Total	%		Urine	Faeces	Total	Total	%
1	Normal	0.3309	0.0311	0.1034	0.1345	0.1964	59.3	0.4107	0.1545	0.1325	0.2870	0.1237	30.1
	Latent	0.2512	0.0388	0.0878	0.1266	0.1246	49.6	0.3678	0.1607	0.0876	0.2543	0.1135	30.8
	Active	0.0765	0.0410	0.0000	0.0410	0.0355	46.1	0.1821	0.1210	0.0000	0.1210	0.0614	33.9
	Recovery	0.1960	0.0354	0.0512	0.0866	0.1094	55.9	0.1900	0.0800	0.0438	0.1238	0.0682	82.7
2	Normal	0.3417	0.0620	0.1450	0.2070	0.1347	39.4	0.4715	0.2017	0.2200	0.4217	0.0498	24.7
	Latent	0.3635	0.1480	0.1076	0.2556	0.1079	29.7	0.4300	0.2250	0.1302	0.3552	0.0748	33.2
3	Normal	0.2030	0.0140	0.1322	0.1462	0.0568	28.0	0.2933	0.1090	0.1972	0.3063	0.0130	—
	Latent	0.1270	0.0113	0.1021	0.1134	0.0136	10.9	0.1530	0.0792	0.0831	0.1623	0.0093	—
	Active	0.0570	0.0640	0.0000	0.0640	0.0070	—	0.1100	0.1628	0.0000	0.1628	0.0528	—
	Recovery	0.2030	0.0100	0.1411	0.1511	0.0519	25.0	0.2933	0.0705	0.2324	0.3029	0.0086	—
4	Normal	0.2010	0.0068	0.1021	0.1089	0.0921	45.8	0.2830	0.0925	0.1001	0.1926	0.0904	31.9
	Latent	0.1650	0.0113	0.0786	0.0899	0.0751	45.5	0.2500	0.1074	0.0625	0.1699	0.0801	32.0
5	Normal	0.1690	0.0089	0.0885	0.0974	0.0716	42.4	0.2280	0.0935	0.0870	0.1805	0.0475	20.8
	Latent	0.1350	0.0085	0.0614	0.0699	0.0651	48.2	0.1650	0.0910	0.0390	0.1300	0.0350	21.2
Rabbit													
1	Normal	0.0904	0.0094	0.0304	0.0398	0.0506	56.0	0.0910	0.5737	0.1555	0.7292	0.1718	19.0
	1st injection	0.0474	0.0016	0.0151	0.0197	0.0277	58.1	0.4500	0.2870	0.0780	0.3650	0.0850	18.9
	2nd "	0.0242	0.0041	0.0085	0.0126	0.0116	47.9	0.2002	0.1403	0.0295	0.1698	0.0304	15.2
2	Normal	0.0185	0.0092	0.0061	0.0153	0.0032	17.3	0.1802	0.1413	0.0188	0.1601	0.0201	11.1
	1st injection	0.0700	0.0073	0.0236	0.0309	0.0391	55.8	0.6660	0.3000	0.1270	0.4270	0.2390	35.9
	2nd "	0.0201	0.0022	0.0070	0.0092	0.0109	54.2	0.1800	0.1168	0.0464	0.1182	0.0618	34.3
3	Normal	0.1140	0.0120	0.0401	0.0521	0.0619	45.7	1.1310	0.7632	0.2195	0.9827	0.1513	13.3
	1st injection	0.0380	0.0039	0.0128	0.0167	0.0213	56.6	0.3868	0.2608	0.0740	0.3318	0.0480	12.4
	2nd "	0.0282	0.0047	0.0096	0.0143	0.0139	49.3	0.2600	0.2015	0.0325	0.2310	0.0260	10.0
4	Normal	0.0370	0.0201	0.0122	0.0323	0.0047	12.6	0.3600	0.2888	0.0408	0.3296	0.0304	8.4
	1st injection	0.1135	0.0289	0.0305	0.0594	0.0541	47.6	1.1400	0.7856	0.1740	0.9596	0.1813	15.9
	2nd "	0.0224	0.0030	0.0054	0.0011	0.0110	49.6	0.2196	0.1380	0.0444	0.1824	0.0462	21.0
5	Normal	0.0342	0.0118	0.0070	0.0188	0.0054	22.3	0.2002	0.1478	0.0286	0.1764	0.0238	11.9
	3rd "	0.0242	0.0181	0.0066	0.0247	0.0005	—	0.2002	0.1563	0.0295	0.1768	0.0234	11.7

unchanged or slightly increased after the first injection of guanidine but diminished after the second and third doses.

Cybulski [1906], Haskins and Gerstenberger [1909] and Salvesen [1923] found a decreased retention of calcium in tetany, the increased output being both by urine and faeces. Cooke [1910] failed to obtain an increase of calcium either in the urine or faeces and maintained that tetany was produced by an altered salt equilibrium brought about by accumulation of acid. Greenwald [1929] although he upholds the low calcium theory of the causation of tetany, found with Hastings and Murray [1921] a retention of calcium. Several investigators have mentioned the possibility of acid substances being involved in the increased loss of lime. Thus Salvesen attributed the loss of lime to the high phosphate content of the blood. Table VI shows the close relationship between the excess excretion of total calcium and that of amino-acids. The excess amino-acid/excess calcium ratio in the parathyroidectomy series varied from 10.3 to 10.8 in all but one instance and in all but two of the guanidine series from 9.5 to 11.0. In the exceptions it will be noted that there was an actual increase in the retention of calcium and a reduction in the output of amino-acids. The other results which show such a close correlation, seem to

Table VI. *Calcium and amino-acid excretion.*

Cat	Period	Excess Ca over normal excretion in g.	Excess amino-acid over normal excretion in g.	Amino-acid Calcium
1	Latent	0.0245	0.25804	10.53
	Active	0.0099	0.10210	10.31
2	Latent	0.0220	0.23805	10.82
3	Latent	0.0220	0.23730	10.78
	Active	0.0230	0.23805	10.35
4	Latent	0.0004	0.00416	10.4
5	Latent	-0.0080 (retention)	-0.01052 (retention)	1.32
Rabbit				
1	1st injection	-0.0058	-0.0227	3.91
	2nd "	0.0020	0.0208	10.4
	3rd "	0.0071	0.0701	9.9
2	1st "	0.0031	0.0314	10.1
3	1st "	-0.0007	-0.01017	14.5
	2nd "	0.0014	0.01409	10.6
	3rd "	0.0154	0.14665	9.5
4	1st "	-0.0004	-0.00392	9.8
	2nd "	0.0061	0.06100	10.0
	3rd "	0.0120	0.13280	11.0

indicate that there is some etiological relationship between the excess production of amino-acids and the decreased retention of calcium. These findings lead one tentatively to suggest that the decalcification of the bones which has been shown radiologically to occur after parathyroidectomy [Erdheim, 1911] may be due to the formation of amino-acids in excessive amounts.

Phosphorus.

Greenwald [1922] found a decreased retention of phosphorus during the latent stage following parathyroidectomy and a marked increase during the active period. Recently Greenwald [1929] concluded that the only two constant changes following parathyroidectomy were a fall in serum-calcium and a retention of phosphorus and maintained that the phosphorus is precipitated in the tissues as calcium phosphate. The increase in blood-phosphorus he attributed to a breakdown of lipoids and proteins containing phosphorus. Esau and Stoland [1930] have elaborated this theory on the basis of the recent work carried out on the relationship of phosphocreatine to muscle metabolism.

The results in connection with phosphorus metabolism are given in Table V. They indicate that after parathyroidectomy there is but slight change in the retention of phosphorus. After injection of guanidine the percentage retention of phosphorus is diminished, the diminution becoming more marked after succeeding injections. Owing to the marked reduction in the intake it may be urged that the decrease in the percentage is a result not of abnormal metabolic processes but simply of the smaller amounts ingested. In rabbit No. 4, however, the intake of phosphorus was increased in the periods following the second and third injections and despite this the absolute amount of phosphorus retained was appreciably less. This lends support to the

view that there is an increased catabolism of phosphorus-containing substances as a result of guanidine poisoning.

In view of the diminished retention of calcium and the fact that four-fifths of the calcium of the body is united with phosphorus it seems at first glance strange that the phosphorus retention should not run parallel with that of calcium. It has to be remembered, however, that whereas practically the sole depôt of calcium is bony tissue, considerable amounts of phosphorus are also found in combination with lipoids and nitrogenous substances.

On the basis of the results recorded here it seems possible to formulate a hypothesis as to the chemical pathogenesis of tetany produced either by parathyroidectomy or by injection of guanidine. The primary effect in both cases is to produce an abnormal protein catabolism resulting in the formation of some toxic amino-compound which immediately combines with an organic chlorine compound. Simultaneously there occurs an excessive catabolism of phosphorus-containing substances and formation of large amounts of amino-acids. The effect on calcium and phosphorus suggests that the excess amino-acid carries away calcium from the bone leaving phosphorus either to be excreted or to form a phosphocreatine compound. Whether the extra phosphocreatine formed in the body is the result of excessive guanidine formation following parathyroidectomy we have no means of deciding from the data presented here. It is certainly suggestive that injection of guanidine produces metabolic results bearing a striking resemblance to those following removal of the parathyroids.

There is not the same gradation in the results observed following guanidine injection as there is after parathyroidectomy. It must be remembered, however, that in the latter case the abnormality produced leads either to continuous production of some abnormal substance or to an equally continuous process of excessive protein breakdown. In guanidine poisoning under the conditions of the experiment there is but one massive injection of poison. If this dose is too great death is likely to ensue, whereas if the body is able to deal with it a return to normal is likely to follow provided a second injection is not given too quickly. The fact that chlorine is retained in such large amount after even a single injection of guanidine, and this despite the fact that there is comparatively little upset of nitrogen metabolism, suggests that guanidine is detoxicated by a chlorine compound. If the available chlorine is sufficient to detoxicate the guanidine there will be but little disturbance. This would explain the variability in the results recorded of guanidine injection. The immediate effect at any rate seems to depend not simply on the amount of guanidine injected but on the amount of available chlorine. The fact that succeeding doses of guanidine of the same amount as the first produce effects quite out of proportion to the first indicates that the amount of available chlorine progressively decreases.

The actual onset of active tetany, if this hypothesis be correct, does not occur until the amount of toxic substance exceeds the amount of available

chlorine. As already mentioned this would explain the conditions of so-called tetania chloropriva. Furthermore, the carrying away of the calcium by the excess amino-acids would account for the low serum-calcium. The rise in inorganic phosphorus which tends to occur could be explained by the extra production of acid-soluble phosphorus resulting from abnormal nucleoprotein catabolism.

SUMMARY.

Following parathyroidectomy or injection of guanidine there occur the following metabolic disturbances.

A. *Chlorine*. There is a marked retention of chlorine in the tissues. In the latent tetany stage there is an increase of volatile chlorine both in the blood and tissues while in the active period there is almost complete absence of volatile chlorine.

B. *Nitrogen*. There is an increased catabolism of tissue protein associated with a rise in the urinary output of total nitrogen, amino-acids and purine-nitrogen and a fall in creatinine and ammonia.

C. *Calcium*. The percentage retention is decreased as a result of relative excess in the urinary excretion. There is a correlation between the excess of calcium and amino-acids excreted.

D. *Phosphorus*. The retention is either slightly diminished or unchanged.

It is suggested (1) that the primary effect in both parathyroidectomy and guanidine poisoning is an abnormal breakdown of tissue proteins and that the changes in calcium, phosphorus and chlorine are secondary to the disturbance in nitrogen metabolism, and (2) that the appearance of active tetany is in part at any rate due to a relative deficiency of chlorine compared with some toxic amino-compound occurring in the course of protein breakdown.

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REFERENCES.

- Albertoni (1914). *Centr. exp. Med.* **5**, 163.
Berkeley and Beebe (1909). *J. Med. Res.* **20**, 149.
Burns (1917). *Quart. J. Exp. Physiol.* **10**, 361.
Carlson and Jacobson (1911). *Amer. J. Physiol.* **28**, 633.
Cathcart, Paton and Pembrey (1925). *Practical physiology*, p. 332. (E. Arnold and Co.)
Cooke (1910). *J. Exp. Med.* **12**, 45.
—— (1911). *J. Exp. Med.* **13**, 439.
Cybulski (1906). *Monatsh. Kinderheilk.* **5**, 1906.
Erdheim (1911). *Z. Path.* **7**, 175.
Esau and Stoland (1930). *Amer. J. Physiol.* **92**, 1.
Falta and Kahn (1912). *Z. klin. Med.* **74**, 105.
Grant (1922). *Arch. Int. Med.* **80**, 355.
Greenwald (1913). *J. Biol. Chem.* **14**, 363.
—— (1922). *J. Biol. Chem.* **54**, 285.
—— (1929). *J. Biol. Chem.* **82**, 531.
Haskins and Gerstenberger (1909). *Cleveland Med. J.* **8**, 671.
Hastings and Murray (1921). *J. Biol. Chem.* **46**, 233.
Iversen and Hansborg (1922). *Acta Med. Scand.* **57**, 95.
Justschenko (1913). *Biochem. Z.* **48**, 61.
MacCallum, Lintz, Vermilye, Leggett and Boas (1920). *Bull. Johns Hopkins Hosp.* **31**, 1.
—— and Voegtlin (1911). *J. Pharm. Exp. Ther.* **2**, 447.
McCrudden (1911). *J. Biol. Chem.* **10**, 187.
Morris and MacRae (1931). Unpublished observations.
—— and Morris (1930). *Biochem. J.* **24**, 1710.
Palladin and Grilliches (1924). *Biochem. Z.* **146**, 258.
Parhon, Ballif and Derevici (1926). *Compt. Rend. Soc. Biol.* **95**, 786.
Paton and Findlay (1917). *Quart. J. Exp. Physiol.* **10**, 315.
Rockemann (1923). *Arch. Kinderheilk.* **72**, 161.
Salvesen (1923). *Acta Med. Scand.* **60**, Suppl. 6.
Underhill, Tileston and Bogert (1922). *J. Med. Res.* **1**, 724.
Van Slyke (1923). *J. Biol. Chem.* **58**, 523.
Wilson, Stearns and Thurlow (1915). *J. Biol. Chem.* **23**, 89.

XC. THE ENZYMIC HYDROLYSIS OF LECITHIN.

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SINCE the original isolation by Thudichum [1874] of lecithin from brain tissue, the study of this substance in relation to its chemistry and to its physiological significance has been a subject of increasing interest. The work of Willstätter and Lüdecke [1904], of MacLean [1912, 1915] and of Levene and his co-workers [1918, 1921, 1922, 1927] on the nature of its cleavage products on hydrolysis and on the preparation of the phosphatide in a pure state has thrown much light on the chemical nature of lecithin. Within recent years Grün and Limpächner [1927] have succeeded in synthesising, from distearin, phosphoric anhydride and choline, interesting substances, which, if not identical with the naturally occurring phosphatides, are certainly remarkably like them in their physical and chemical properties.

The rôle of phosphatides in metabolism has been a matter of considerable speculation and much experiment. McCollum *et al.* [1912] have shown that the nature of the fatty acid radicals in the lecithin of eggs changes with, and is presumably dependent on, the nature of the fats contained in the diet. Levene and Simms [1921] have shown that, as in the case of the fats themselves, the fatty acid radicals of the lecithin isolated from liver are more unsaturated than those of lecithin from brain or eggs. The view has been repeatedly advanced that the phosphatides play an important part in the metabolism of the fats as a whole. The fact that lecithin is more readily oxidised and hydrolysed than neutral fat and that the lecithin-fat of the liver, where desaturation is thought to take place, is more unsaturated than the fat of other parts of the body, may be taken as evidence supporting this view. Bloor and Knudson [1916, 1917] have observed a definite increase in the phosphatide content of both corpuscles and plasma following the ingestion of a meal containing fat, and Sinclair [1929] has found that the ingestion of cod-liver oil increases the iodine value of the lipid fatty acids of the mucosa.

The importance of phosphatides as essential fat is well illustrated in the brain. This organ which contains the largest amount of phosphatide loses none of it during starvation, and the inference that this form of fat is essential to the life of the organ would seem justifiable.

The rôle of the body enzymes in relation to lecithin has received considerable attention from various workers. Bokay [1877] investigated the action of the intestinal lipase, and since then many publications dealing with the hydrolysis of lecithin by intestinal and ricinus lipase have appeared. Some of the more recent work in this connection is due to Contardi and Latzer [1927], who studied the hydrolysis of lecithin cadmium chlorides, and by Paal [1929] who found the lipase cleavage of lecithin to give parallel results for the liberation of fatty acid and choline.

Since the study of body phosphatases during recent years has led to such important results in the elucidation of bone formation [Robison, 1923; Robison and Soames, 1924, 1925; Martland and Robison, 1926, 1927] and other physiological processes in health and disease [Kay, 1926, 1928, 1930; Neuberg, 1918, 1926, 1928; Meyerhof, 1928], it seemed possible that a study of the action of an enzyme which would hydrolyse lecithin to liberate phosphate might lead to a better understanding both of the chemical nature of this substance and of its significance in the body processes.

Kay [1926] noted the presence of a feeble "lecithinase" in an extract of kidney, which slowly hydrolysed phosphate from lecithin at 37° and had an optimum p_H of 7.4, about that of the tissues. He regards this as being a different enzyme from kidney phosphatase, the optimum p_H of which is 8.9, and which he has some experimental evidence to show is identical with intestinal and bone phosphatase. The latter Martland and Robison [1926] state to be without action on phosphatides.

The action of a phosphate-liberating enzyme on lecithin is more complex than that of the bone phosphatase on hexose- or glycerophosphate, since it involves the cleavage of both choline and the glycerol residue. This, however, might be said of the enzymic hydrolysis of any secondary ester of phosphoric acid where two esterified groups must be separated in order to liberate free phosphoric acid; and the optimum p_H for hydrolysis of such esters, 7.7, as found by Asakawa [1929]¹, is remarkably near that reported in a former investigation [King and Page, 1930] for the enzyme contained in extracts of intestine and kidney which hydrolysed lecithin most actively at p_H 7.5-7.6. This question of the relation of the enzymes responsible for the hydrolysis of primary and secondary esters of phosphoric acid and of lecithin will be dealt with more fully in a later communication. The investigation detailed in this paper consists of a study of the liberation of phosphoric acid from lecithin under various conditions and of the occurrence and distribution of the enzyme responsible for this hydrolysis in a large number of animal tissues.

EXPERIMENTAL.

That there is an enzyme present in autolysing tissues which is responsible for the liberation of free phosphate from compounds other than those usually

¹ Asakawa investigated the action of kidney phosphatase on a large number of primary and secondary esters of phosphoric acid, including diphenyl, dicresyl, diglyceryl and diethyl phosphate.

referred to as "acid-soluble" may be inferred from the fact that the "free phosphate" in a trichloroacetic acid extract of a tissue made after a few days' autolysis is greater than the "total phosphate" present in such an extract of the fresh tissue. This is shown by Table I where are given the amounts, at different times, of free and total phosphate present in the trichloroacetic acid extracts of aliquot portions of composite samples of rabbit liver and kidney, which were ground with sand and water and allowed to autolyse in the presence of chloroform. It is probable that this extra phosphate from a non-acid-soluble source is largely derived from lecithin, and it seemed worth while to investigate the enzyme responsible for this hydrolysis.

Table I.

Days	Kidney		Liver	
	Free phosphate mg. P in 1 cc. of trichloroacetic acid extract	Total phosphate	Free phosphate mg. P in 1 cc. of trichloroacetic acid extract	Total phosphate
0	0.113	0.128	0.211	0.265
1	0.115	—	0.242	—
4	0.131	—	—	0.269
5	0.134	0.134	0.271	—
7	0.141	—	0.289	—
9	0.148	—	0.298	0.306
11	0.149	0.150	0.304	—
13	0.153	—	0.318	—
15	0.155	—	—	—

MacLean [1927] has pointed out that probably in none of the researches on the action of enzymes on lecithin has pure lecithin been used. The obvious impurity of commercial lecithin, must, of course, complicate any results where this material is used as a substrate. One brand of egg-lecithin, which was tried, was only 40 % precipitable by cadmium chloride, and this precipitate, when freed from cadmium and dissolved in ether, gave only a very small precipitate when poured into acetone.

With purified lecithins from different sources, however, there seems to be very little difference in the amount of hydrolysis by the phosphate-splitting enzyme. Table II contains the results of the hydrolysis of three different lecithins by different amounts of enzyme solutions prepared by extracting ground-up beef kidney and small intestine of rabbit with 20 times their weight of distilled water saturated with chloroform. The lecithins used in these experiments were prepared from eggs, from brain, and from Kahlbaum's lecithin, by the method of Levene and Rolf [1927]. Emulsions of the lecithins were made by running 1 volume of 2.5 % alcoholic solution of the lecithin into 10 volumes of hot borate buffer (Palitzsch) at p_H 7.5, with vigorous shaking. This gives a permanent emulsion of lecithin which contains 0.5 mg. of phosphorus in 5 cc. and practically no free phosphate. For the enzymic hydrolysis a series of test-tubes containing 5 cc. of lecithin emulsion and various amounts of the tissue extract were incubated in the presence of a drop of chloroform and toluene for 48 hours at 37.5°. Controls consisted of

5 cc. of borate buffer *plus* the same amounts of extract. Previous work [King and Page, 1930] had shown that there is practically no increase in the free phosphate of a lecithin emulsion itself when incubated at this temperature, so controls of lecithin emulsion *plus* water were omitted. At the end of the 48 hours the hydrolysis was stopped by the addition of 25 % trichloroacetic acid, and the inorganic phosphorus of the filtrate estimated by the Martland and Robison [1924] modification of the Briggs colorimetric procedure. The experiments were done in duplicate and the figures are the average of duplicate determinations.

Table II. *Hydrolysis of different lecithins by varying amounts of tissue extract.*

cc. of extract	Free P extract and buffered lecithin mg.	Free P extract and buffer mg.	Increase in free P mg.
(1) Rabbit intestinal extract and egg-lecithin:			
1	0.208	0.184	0.024
3	0.698	0.545	0.153
5	1.137	0.937	0.200
10	2.280	1.840	0.440
Rabbit intestinal extract and purified Kahlbaum's lecithin:			
1	0.240	0.199	0.041
3	0.714	0.560	0.154
5	1.154	0.948	0.206
10	2.191	1.837	0.354
Rabbit intestinal extract and brain-lecithin:			
1	—	0.161	—
3	0.647	0.546	0.101
5	1.108	0.923	0.185
10	2.243	1.833	0.410
(2) Beef kidney extract and egg-lecithin:			
1	0.176	0.162	0.014
3	0.589	0.462	0.127
5	1.001	0.791	0.210
10	1.951	1.561	0.390
Beef kidney extract and purified Kahlbaum's lecithin:			
1	—	0.162	—
3	0.592	0.462	0.130
5	0.981	0.791	0.190
10	1.869	1.561	0.308

It has been shown by Kay [1926] that different buffer solutions have an effect on the amount of hydrolysis of glycerophosphate by kidney phosphatase. The hydrolysis in presence of borate buffer is always considerably less than that obtained when glycine buffer is used. No such effect as this was noticed in the enzymic hydrolysis of lecithin. The amount of hydrolysis in the presence of borate buffer at p_H 7.5 was practically the same with intestinal and kidney extracts as in the absence of borate, the lecithin emulsion being made up in distilled water and the mixture of lecithin and extract adjusted to p_H 7.5 by the careful addition of acid and alkali. The veronal buffer of Michaelis [1930] is very useful for the range covered by phosphate-hydrolysing enzymes, but appears to have no advantage over the borate mixture of Palitzsch in the

hydrolysis of lecithin, as the extent of hydrolysis appears to be very nearly the same in the presence of either buffer. Thus 2 cc. of rabbit intestinal extract liberated 0.115 mg. of phosphorus from 5 cc. of lecithin emulsion in the presence of veronal buffer and 0.105 mg. in the presence of borate; and 2 cc. of rabbit kidney extract liberated 0.111 mg. phosphorus with the veronal, and 0.103 mg. with the borate buffer during 48 hours' hydrolysis.

The hydrolysis of lecithin in borate buffer at varying times and at different hydrogen ion concentrations is illustrated in Fig. 1. Mixtures of 8 cc. of beef

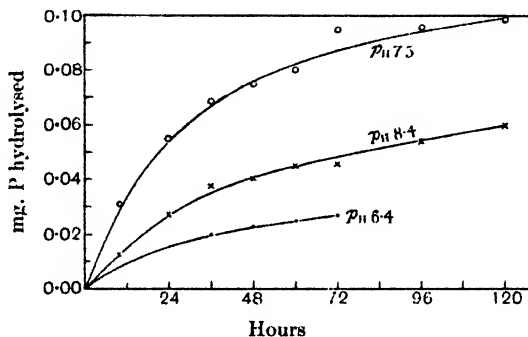


Fig. 1. Hydrolysis of lecithin at different p_H .

kidney extract, 40 cc. of 0.25 % lecithin emulsion (in water) and 40 cc. of borate buffer (of the desired p_H) were kept in the thermostat over a period of several days. 6 cc. portions were removed every 12 hours into 1 cc. of 25 % trichloroacetic acid and the inorganic phosphorus estimated in the filtrate. At the optimal p_H there was a rapid increase in the free phosphate during the first 60 hours, and thereafter a decrease in the rate of hydrolysis until equilibrium seemed to be approached between the fourth and fifth days.

As about 75 % of the hydrolysis had occurred during the first 48 hours, this was adopted as the standard period of time for comparative hydrolysis of lecithin by different tissue extracts. The method used for estimating the amount of the lecithin-hydrolysing enzyme in any tissue is as follows. The sample, freed from adhering tissue, is weighed, ground with sand and transferred with 20 times its weight of water saturated with chloroform to an Erlenmeyer flask. It is allowed to stand at room temperature, with occasional shaking, for 48 hours, when the mixture is filtered through cotton wool, adjusted to p_H 7.5 and preserved with a little toluene in the refrigerator. Longer periods of extraction, frequent grinding of the mixture or repeated extraction with smaller quantities of water do not appear to increase appreciably the amount of enzyme extracted. In the case of kidneys and similar tissues, the whole organ is taken, or it is minced up finely with scissors and an aliquot portion used for grinding and extraction. Intestines are freed from fat and extraneous tissue, split open lengthwise and gently washed out with a little water before mincing. Bones are freed as far as possible from muscle.

split open and freed from marrow before weighing. (Owing to the low "lecithinase" activity of bone, only 10 times the weight of water is used to make the extract instead of 20 times as in the case of other tissues.) To test the enzymic potency of an extract, two test-tubes containing 5 cc. of Palitzsch's borate buffer solution at p_H 7.5, 5 cc. of the 0.25 % lecithin emulsion, and 2 cc. of the tissue extract are incubated at 37.5° in the presence of a drop of chloroform and toluene. Controls consisting of buffer and extract and buffer and lecithin are prepared and incubated in the same way. The amount of free phosphate in the buffer and lecithin tubes is usually very small and may be neglected. At the end of the 48 hours the hydrolysis is stopped by the addition of 3 cc. of 20 % trichloroacetic acid, and the inorganic phosphorus is estimated in the filtrate. The amount of phosphate liberated is taken as a measure of the enzymic activity of the extract and hence of the tissue.

The hydrolysis of lecithin to yield free phosphate probably occurs in two stages, the first of which should yield either a glyceridephosphate or choline phosphate, while the second would involve the cleavage of either or both of these compounds to give free phosphate. Since the glyceridephosphate would probably, and choline phosphate almost certainly, be more soluble than lecithin in trichloroacetic acid solution, it was considered worth while to estimate the total as well as the inorganic phosphorus in the trichloroacetic acid filtrates. In general, the increase in total acid-soluble phosphorus of the tubes containing lecithin and extract over that of the controls ran parallel with, and was slightly greater than, the increase in the inorganic phosphorus.

While phosphatase has an optimum p_H of 8.9 for its action on glycerophosphoric acid, quite different from that of lecithinase, it is conceivable that in some way it may be concerned in the liberation of inorganic phosphorus from lecithin. It was therefore interesting to determine whether the phosphatase activity of the extracts ran parallel with the phosphate-hydrolysing enzyme for lecithin. Such did not appear to be the case. The extracts of bone, which generally showed a high phosphatase activity, were usually without any appreciable action on lecithin. The intestinal and kidney extracts, on the other hand, had a high enzymic activity for both glycerophosphate and for lecithin, but there was considerable variation in the values obtained and no relation could be seen between the phosphatase and lecithinase activities. The method used for determining the phosphatase activity of the extracts consisted in placing 5 cc. of 0.3 % sodium β -glycerophosphate, 5 cc. of glycine buffer and 0.5 cc. of extract (all of p_H 8.9) in each of four test-tubes. 2 cc. of 25 % trichloroacetic acid were added immediately to two of the tubes and the other two were kept at 37.5° in the presence of toluene for exactly 2 hours, and 2 cc. of trichloroacetic acid then added. The increase in free phosphate was taken as the measure of phosphatase activity.

Table III contains the results of a considerable number of determinations of the lecithinase and phosphatase activity of mammalian and avian tissues.

Table III. *Hydrolysis of lecithin and of glycerophosphate by tissue extracts.*

Free P hydrolysed from lecithin mg.	Total P hydrolysed from lecithin (acid-soluble) mg.	Free P hydrolysed from glycerophosphate mg.	Free P hydrolysed from lecithin mg.	Total P hydrolysed from lecithin (acid-soluble) mg.	Free P hydrolysed from glycerophosphate mg.
Pig kidney			Rabbit lung		
0.030	—	0.223	0.009	0.006	0.065
0.020	—	—	0.012	0.036	0.105
			0.005	0.015	0.074
Beef kidney			Rabbit aorta		
0.000	—	—	0.000	0.010	0.000
0.031	—	0.179	0.000	0.009	0.000
0.084	—	—			
Beef suprarenal			Rabbit skeletal muscle		
0.001	—	—	0.003	0.013	0.005
0.012	—	—	0.001	0.029	—
			0.000	0.007	0.011
Dog kidney			0.002	0.005	0.002
0.024	—	0.151			
0.049	—	0.174	Rabbit heart		
			0.003	0.024	0.008
Dog intestine			0.002	0.002	0.009
0.024	—	0.223	0.002	0.013	0.010
			0.005	0.006	0.013
Human baby (acute intestinal diarrhoea)			Rabbit large intestine		
Large intestine			0.009	0.010	0.014
0.006	0.006	0.056	0.017	0.150	0.024
Small intestine			0.005	0.011	0.019
0.012	0.012	0.009	0.006	0.005	0.015
Human baby (stillborn)			Rabbit small intestine		
Small intestine			0.021	—	0.075
0.012	—	0.176	0.008	—	0.104
			0.057	0.008	0.268
Kidneys			0.010	0.010	0.061
0.013	—	0.003	0.020	0.061	0.074
			0.016	0.227	0.236
Rabbit liver			0.027	—	0.379
0.011		0.058	0.020	—	0.040
0.022	0.059	0.104	0.030	0.086	0.219
0.006	0.006	0.048	0.117	—	0.255
			0.137	—	0.114
Rabbit suprarenals			Rabbit kidney		
0.008	0.008	0.006	0.006	—	0.251
0.008	0.032	0.072	0.012	0.063	0.214
0.011	0.020	0.012	0.029	0.036	0.314
0.003	0.004	0.018	0.009	0.030	0.172
			0.035	—	0.252
Rabbit pancreas			0.024	—	0.112
0.005	0.049	0.001	0.049	0.062	0.224
0.009	0.059	0.008	0.098	—	0.227
0.006	0.006	0.003	0.014	0.060	0.179
Rabbit spleen			Rabbit bone		
0.015	0.018	0.097	0.021	—	0.134
0.021	0.022	—	0.010	—	0.231
0.034	0.042	—	0.024	0.135	0.307
0.029	0.039	0.027	0.007	0.007	0.286
			0.001	0.013	0.309
Rabbit testes			0.012	—	0.174
0.008	0.011	0.316	0.024	—	0.145
0.023	0.024	0.336	0.009	—	0.112
			0.003 (old)	0.022	0.052
Rabbit ovaries			0.029 (young)	0.073	0.536
0.006	0.009	0.101	0.076 (rickets)	0.252	0.267
0.010	0.021	0.059			
			Chicken liver		
Rabbit brain			0.031	0.038	0.117
0.006	0.012	0.021	0.021	0.032	0.105
0.011	0.043	0.022	0.021	0.016	0.088
0.023	0.048	0.033	0.005	0.016	0.028
0.008	0.010	0.020			

Table III (contd.).

Free P hydrolysed from lecithin mg.	Total P hydrolysed from lecithin (acid-soluble) mg.	Free P hydrolysed from glycerophosphate mg.	Free P hydrolysed from lecithin mg.	Total P hydrolysed from lecithin (acid-soluble) mg.	Free P hydrolysed from glycerophosphate mg.
Chicken gizzard			Chicken cardiac muscle		
0-004	0-007	0-004	0-001	0-002	0-018
0-001	0-017	0-004	0-004	0-017	0-008
0-000	—	0-007	0-008	0-016	0-013
0-008	0-017	0-009			
Chicken pancreas			Chicken large intestine		
0-006	0-019	0-012	0-031	0-065	0-062
0-002	0-031	0-014	0-005	0-031	0-021
0-041	0-083	0-012	0-012	0-068	0-017
0-022	0-021	0-005	0-021	0-033	0-014
Chicken spleen			Chicken small intestine		
0-010	0-054	0-018	0-023	0-039	0-027
0-020	0-069	0-012	0-015	0-091	0-030
0-001	0-020	0-009	0-019	0-189	0-035
Chicken proventriculus			0-046	0-056	0-051
0-013	0-031	0-015	0-012	0-125	0-066
0-017	0-038	0-029	0-008	0-062	0-032
0-002	0-037	0-010	0-006	0-110	0-034
Chicken testes			0-006	0-076	0-018
0-040	0-061	0-096	0-007	0-036	0-050
0-012	0-024	0-047			
0-008	0-010	0-008	Chicken kidney		
0-013	0-017	0-055	0-022	—	0-230
0-007	0-016	0-039	0-017	0-022	0-059
0-011	0-030	0-051	0-031	0-068	0-138
0-016	0-059	0-027	0-038	0-178	0-183
Chicken brain			0-053	0-054	0-344
0-010	0-018	0-036	0-016	0-043	0-277
0-008	0-027	0-041	0-012	0-023	0-227
0-006	0-011	0-024	0-032	—	0-256
0-015	0-015	0-016	0-036	0-079	0-260
Chicken lung			0-020	0-058	0-254
0-006	0-017	0-023	0-026	0-032	0-196
0-001	0-007	0-011	Chicken bone		
0-002	0-002	0-011	0-020	—	0-331
0-011	0-017	0-054	0-004	—	0-046
Chicken blood vessels			0-000	0-005	0-035
0-014	0-020	0-037	0-003	0-035	0-185
0-001	0-004	0-005	0-007	0-010	0-157
0-009	0-014	0-039	0-011	0-024	0-358
Chicken skeletal muscle			0-021	0-024	0-387
0-005	0-007	0-008	0-016	—	0-178
0-002	0-017	0-004	0-028	—	0-076
—	0-008	0-002	0-001	0-044	0-085
0-007	0-007	0-005	0-000	0-037	0-110
			0-003	0-008	0-132

Inactivation of the enzyme.

When an active tissue extract is kept at body temperature at different hydrogen ion concentrations, a marked destruction of the enzyme occurs at reactions which are not near neutrality. Fig. 2 illustrates the results which were obtained on the inactivation of an extract of rabbit kidney. 10 cc. quantities of the extract were adjusted to different p_H by the careful addition of a few drops of $N/10$ acid or alkali. After being left in the thermostat at 38° for 24 hours, the samples were adjusted to p_H 7.6 by adding more acid or alkali. Water was then added to all of them to bring the volume to 12 cc.

The lecithinase and phosphatase activities were determined on 2 cc. and 0.5 cc. portions, respectively, and the results compared with the activity of the original extract. Between p_H 7.0 and 8.0 there appeared to be very little loss of activity towards either lecithin or glycerophosphate. Above p_H 8, and below p_H 6, however, there was a marked diminution in the activity of the extracts.

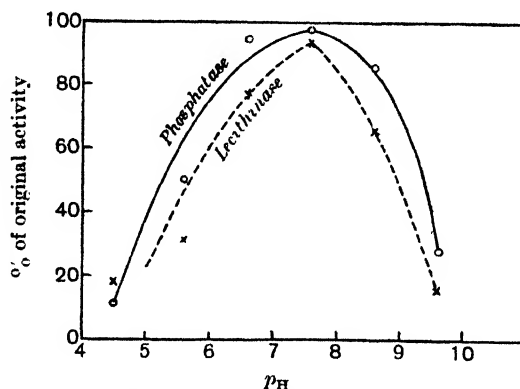


Fig. 2. Inactivation of the enzyme.

Hydrolysis of lecithin at different temperatures.

The optimum temperature for the hydrolysis of lecithin appears to be that of the body. Incubation of hydrolysing mixtures at lower or higher temperatures than 38° results in a smaller increase in the free and total acid-

Table IV. *Hydrolysis of lecithin at different temperatures.*

	Days	Free P mg.	Increase in free P mg.	Total acid- soluble P mg.	Increase in total acid- soluble P mg.
(1) Temperature 11°	0	0.220	—	0.244	—
	1	0.221	0.001	0.245	0.001
	2	0.223	0.003	0.246	0.012
	3	0.224	0.004	0.251	0.007
	4	0.227	0.007	0.254	0.010
	5	0.231	0.011	0.266	0.022
(2) Temperature 23°	0	0.220	—	0.244	—
	1	0.224	0.004	0.253	0.009
	2	0.235	0.015	0.275	0.031
	3	0.245	0.025	0.278	0.034
	4	0.254	0.034	0.285	0.041
	5	0.261	0.041	0.302	0.058
(3) Temperature 38°	0	0.220	—	0.244	—
	1	0.232	0.012	0.262	0.018
	2	0.252	0.032	0.272	0.028
	3	0.261	0.041	0.298	0.054
	4	0.276	0.056	0.296	0.052
	5	0.288	0.068	0.314	0.070
(4) Temperature 57°	0	0.220	—	0.244	—
	1	0.221	0.001	0.261	0.017
	2	0.226	0.006	0.261	0.017
	3	0.231	0.011	0.282	0.038
	4	0.240	0.020	0.278	0.034
	5	0.244	0.024	—	—

soluble phosphorus. Two test-tubes, each containing 20 cc. of buffer (p_H 7.5), 20 cc. of lecithin emulsion and 10 cc. of kidney extract, were kept in thermostats at different temperatures for a period of 5 days, 8 cc. being withdrawn each day into 2 cc. of 25 % trichloroacetic acid for the determination of free and total phosphorus (Table IV).

The effect of hydrolysis products on the rate of hydrolysis.

If the velocity constants for a unimolecular reaction,

$$k = \frac{1}{t} \log \frac{a}{a-x},$$

are calculated from the data of hydrolysis experiments, it is seen that there is a very marked difference in the behaviour of the hydrolysing mixtures according to the enzyme preparation used. For some kidney preparations the values for k are fairly constant over a period of several days. For others, and especially for intestinal preparations, the reaction velocities show a marked and steady decline and soon reach values which are much smaller than those for the original rates of hydrolysis. The data for four experiments which were carried out to test this point are contained in Table V.

Table V. *Rate of hydrolysis of lecithin.*

Time hrs.	Free P hydro- lysed mg.	<i>k</i>	Time hrs.	Free P hydro- lysed mg.	<i>k</i>	Total acid- soluble P hydro- lysed mg.	<i>k</i>
Rabbit kidney extracts:							
24	0.021	0.000778	8	0.017	0.00186	0.037	0.00418
48	0.034	0.000648	24	0.058	0.00223	0.093	0.00372
72	0.062	0.000803	32	0.073	0.00214	0.109	0.00333
96	0.083	0.000824	48	0.103	0.00209	0.143	0.00304
120	0.091	0.000731	59	0.122	0.00206	0.170	0.00305
144	0.106	0.000722	72	0.127	0.00176	0.173	0.00255
168	0.120	0.000714	94	0.136	0.00147	0.170	0.00192
192	0.120	0.000623	Total lecithin-P 0.500 mg.				
216	0.127	0.000592					
240	0.134	0.000567					
Total lecithin-P 0.500 mg.							
Rabbit intestinal extracts:							
24	0.159	0.00693	24	0.012	0.000566	0.031	0.001461
48	0.171	0.00379	48	0.013	0.000302	0.032	0.000759
72	0.191	0.00290	72	0.012	0.000189	0.042	0.000673
96	0.199	0.00229	96	0.015	0.000176	0.051	0.000622
120	0.228	0.00220	120	0.023	0.000214	—	—
144	0.231	0.00187	144	0.024	0.000189	0.069	0.000572
168	0.232	0.00161	168	0.029	0.000196	0.077	0.000553
192	0.233	0.00142	192	0.031	0.000184	0.077	0.000484
216	0.239	0.00130	216	0.033	0.000174	—	—
240	0.244	0.00121	Total lecithin-P 0.400 mg.				
Total lecithin-P 0.500 mg.							
Enzyme solutions dialysed 48 hours against running water.				Enzyme solutions electro dialysed against tap-water till nearly free of inorganic phosphate.			

It would seem that this decline in the values of k is more probably due to the slow destruction of the enzyme in the hydrolysing mixture than to the

accumulation of the products of hydrolysis. The increases of inorganic phosphate in the experiments cited are not sufficiently different in amount to account, on the basis of inhibition due to accumulation of a product of hydrolysis, for the large variations in the values for the velocity constants.

Several attempts were made to ascertain the effect on the rate of hydrolysis of added inorganic phosphate, but such small differences as were observed in the amounts hydrolysed were irregular and could not be attributed to the extra phosphate present.

Since the cleavage of inorganic phosphorus from lecithin also involves the removal of choline, it was of interest to determine what influence, if any, choline had on the amount of hydrolysis as estimated by the liberation of phosphate. Table VI contains the results of an experiment in which choline, in varying amounts, was added to digesting mixtures of lecithin and rabbit kidney extract. The addition of the choline caused no diminution in the amount of phosphorus liberated, and the variations in the values for k between the solutions of different choline content are no greater than the variations in the values for the individual solutions.

Table VI. *Effect of choline on the hydrolysis of lecithin.*

Time hrs.	No choline				0.005 <i>M</i> choline			
	Free P hydro- lysed mg.	k	Total acid- soluble P hydro- lysed mg.	k	Free P hydro- lysed mg.	k	Total acid- soluble P hydro- lysed mg.	k
14	0.020	0.001286	0.031	0.001995	0.020	0.001286	0.030	0.001939
28	0.028	0.000899	0.042	0.001331	0.030	0.000964	0.041	0.001336
38	0.036	0.000863	0.056	0.001362	0.040	0.000960	0.056	0.001369
49	0.055	0.001035	0.054	0.001021	0.055	0.001035	0.057	0.001081
62	0.060	0.000901	0.061	0.000917	0.061	0.000918	0.068	0.001031
75	0.066	0.000822	0.072	0.000906	0.071	0.000892	0.075	0.000948
86	0.067	0.000730	0.084	0.000933	0.074	0.000814	0.082	0.000907
96	0.078	0.000771	0.074	0.000729	0.081	0.000802	0.083	0.000824
110	0.095	0.000836	0.085	0.000737	—	—	—	—
	0.01 <i>M</i> choline				0.04 <i>M</i> choline			
	Free P hydro- lysed mg.	k	Total acid- soluble P hydro- lysed mg.	k	Free P hydro- lysed mg.	k	Total acid- soluble P hydro- lysed mg.	k
14	0.023	0.001499	0.022	0.001421	—	—	0.022	0.001421
28	0.033	0.001071	0.039	0.001268	0.028	0.000899	0.030	0.000968
38	0.041	0.000984	0.054	0.001314	0.040	0.000960	0.048	0.001157
49	0.054	0.001017	0.052	0.000980	0.052	0.000980	—	—
62	0.064	0.000967	0.064	0.000967	0.060	0.000914	—	—
76	0.072	0.000904	—	—	0.067	0.000840	—	—
86	0.076	0.000837	0.078	0.000861	0.072	0.000790	—	—
96	—	—	—	—	0.075	0.000739	0.077	0.000761
110	0.094	0.000827	—	—	—	—	—	—

Total lecithin-P 0.500 mg.

Enzyme solution electrodialysed against tap-water.

The presence of glycerol in the hydrolysing mixtures had no effect in concentrations up to 0.4 *M*, the highest investigated. An experiment similar to the foregoing was carried out with lecithin, kidney extract and varying amounts of added glycerol. A sample equivalent to 0.500 mg. of lecithin-P

was removed from each flask at various periods and the free and total P estimated in the trichloroacetic acid filtrates (Table VII).

Table VII. *Effect of glycerol on the hydrolysis of lecithin.*

Time hrs.	No glycerol		0.01 M glycerol		0.1 M glycerol		0.4 M glycerol	
	Free P hydro- lysed mg.	Total acid- soluble P hydro- lysed mg.	Free P hydro- lysed mg.	Total acid- soluble P hydro- lysed mg.	Free P hydro- lysed mg.	Total acid- soluble P hydro- lysed mg.	Free P hydro- lysed mg.	Total acid- soluble P hydro- lysed mg.
13	0.007	0.019	0.007	0.011	0.003	0.003	0.009	0.010
24	0.019	0.020	0.020	0.027	0.015	0.013	0.014	0.014
35	0.017	0.031	0.018	0.026	0.016	0.016	0.013	—
48	0.030	0.033	0.032	0.024	0.027	—	0.027	0.031
61	0.030	0.050	0.033	0.048	0.027	0.032	0.031	0.039
80	0.042	0.054	0.042	0.054	0.037	—	0.037	0.040
95	—	0.059	0.046	0.058	0.043	0.051	0.041	0.042
105	0.044	0.059	0.046	0.056	—	—	0.041	0.044
120	0.057	0.060	0.057	0.063	0.045	—	0.059	—

SUMMARY.

1. An enzyme capable of hydrolysing lecithin to liberate inorganic phosphate is present in various animal tissues.

2. The optimal hydrogen ion concentration for the action of this enzyme is at about p_H 7.5, while that of phosphatase is at p_H 8.9. For this reason, and because the distribution of "lecithinase" does not correspond with that of phosphatase, they are thought not to be the same enzyme.

3. The relative "lecithinase" activity of different tissues is as follows: (in decreasing order)—kidney, small intestine, spleen, liver, testes, pancreas, large intestine, brain, ovaries, bone, suprarenals, lung, blood vessels, cardiac muscle, skeletal muscle.

The enzyme is fairly stable at a neutral reaction but is rapidly destroyed when kept in an acid or alkaline medium at 38°.

4. Lecithin appears to be hydrolysed most rapidly by the enzyme at body temperature.

5. The addition of inorganic phosphate and choline (products of hydrolysis) and of glycerol to hydrolysing mixtures of lecithin and the enzyme does not appear to affect appreciably either the rate or amount of hydrolysis.

The technical work and colorimetric analyses in connection with this investigation were done by Miss Margaret Shaw.

REFERENCES.

- Asakawa (1929). *J. Biochem. (Japan)*, **11**, 143.
 Bloor and Knudson (1916). *J. Biol. Chem.* **24**, 447.
 ——— (1917). *J. Biol. Chem.* **32**, 337.
 Bokay (1877). *Z. physiol. Chem.* **1**, 157.
 Contardi and Latzer (1927). *Rend. Istit. Lombardo Sci.* 847, 857.
 Grün and Limpächer (1927). *Ber. deutsch. chem. Ges.* **60**, 147, 151.
 Kay (1926). *Biochem. J.* **20**, 791.
 ——— (1928). *Biochem. J.* **22**, 855.
 ——— (1930). *J. Biol. Chem.* **89**, 249.
 King and Page (1930). *Z. physiol. Chem.* **191**, 243.
 Levene (1921). *Physiol. Reviews*, **1**, 327.
 ——— and Rolf (1927). *J. Biol. Chem.* **72**, 587.
 ——— and Simms (1921). *J. Biol. Chem.* **48**, 185.
 ——— (1922). *J. Biol. Chem.* **51**, 285.
 ——— and West (1918). *J. Biol. Chem.* **34**, 175.
 McCollum, Halpin and Drescher (1912). *J. Biol. Chem.* **13**, 219.
 MacLean (1912). *J. Physiol.* **45**; *Proc. xiii: Biochem. J.* **6**, 355.
 ——— (1915). *Biochem. J.* **9**, 351.
 ——— (1927). *Lecithin and allied substances* (Longmans, London).
 Martland and Robison (1924). *Biochem. J.* **18**, 765.
 ——— (1926). *Biochem. J.* **20**, 847.
 ——— (1927). *Biochem. J.* **21**, 665.
 Meyerhof (1928). *J. Gen. Physiol.* **8**, 531.
 Michaelis (1930). *J. Biol. Chem.* **87**, 33.
 Neuberg (1918). *Biochem. Z.* **88**, 432.
 ——— (1926). *Biochem. Z.* **171**, 485.
 ——— (1928). *Biochem. Z.* **199**, 498.
 Paul (1929). *Biochem. Z.* **211**, 244.
 Robison (1923). *Biochem. J.* **17**, 286.
 ——— and Soames (1924). *Biochem. J.* **18**, 740.
 ——— (1925). *Biochem. J.* **19**, 153.
 Sinclair (1929). *J. Biol. Chem.* **82**, 117.
 Thudichum (1874). *Reports of Medical Officer and Local Government Board. New Series*, **3**, 113.
 Willstätter and Lüdecke (1904). *Ber. deutsch. chem. Ges.* **37**, 353.

XCI. THE NATURE OF THE "ETHER REACTION" OF URINE.

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OVER forty years ago Plosz [1890] showed that when urine is strongly acidified with acetic acid and shaken with ether, chloroform or amyl alcohol, the mixture separates into two layers and on the boundary between the two a precipitate of protein is formed. He remarks: "The reaction is given by all urines, since all urines contain protein. The delicacy of the reaction is thereby shown¹. The protein so obtained is not a homogeneous substance since only a portion of it is soluble in acetic acid. Another portion is soluble in water, alkalis, and strongly concentrated solutions of common salt. The fraction insoluble in acetic acid dissolves, after being washed with acetic acid, in dilute alkali, and is again precipitated on addition of acetic acid. It behaves like mucin. Normal urine always contains protein and a body behaving in its reactions like mucin."

Two years later Smith [1892], who evidently had not seen Plosz's paper, described a reaction of urine with ether in which the acidified urine was shaken with ether, and a thick scum was formed as the layers separated. He stated that the scum was most abundant in the urine of those who had a good appetite and good digestion and was usually absent after long fasting or when the diet was greatly restricted.

Oriel and Barber [1930] have found that when acidified urine is shaken with one-fifth of its volume of ether the mixture separates into two layers with a scum between, which varies in extent and consistency according to the health of the person from whom the urine is derived. When the aqueous layer is discarded and alcohol is added to the ether and scum a precipitate is obtained which gives the biuret, Millon, Adamkiewicz and Molisch reactions. They state that the nitrogen content of the precipitate is also variable and in allergic conditions may rise from a few mg. to as much as 300 mg. daily with the onset of an attack, subsiding to its original value as the attack passes. The chief interest of Oriel and Barber's observations, however, lies in their discovery that the precipitate obtained from urine passed during an attack, contains some of the antigen responsible for the attack as shown by skin reactions and by reproduction of the attack when a solution of the precipitate is injected intradermally into the patient from whom it has been derived.

¹ That is, because the boiling test is negative in normal urines.

It appeared to be desirable to investigate the nature of this ether-alcohol precipitate more closely.

Isolation of the crude material.

The material was obtained by treating 500 cc. of urine with 5 cc. of 25 % sulphuric acid, adding 100 cc. of ether, shaking the mixture vigorously in a stoppered separating funnel for a minute and allowing the ether to separate for 15 minutes. The aqueous layer was then discarded and 100 cc. of 93 % alcohol were added to the ether and the scum. The precipitate was allowed to settle and was separated by centrifuging, washed with alcohol and with ether and dried in a desiccator or at 100° as required.

The material could also be obtained by adding to the urine 3 vols. of 93 % alcohol, and collecting the precipitate. The proportion of salts present was, however, much higher in this case.

The quantity of material obtained by means of the ether reaction as described above appeared to be higher in the case of urines from allergic patients than in the case of urines from apparently normal subjects. Thus an asthmatic in an attack yielded 1.067 g. per litre containing 0.047 g. N, whereas men employed on indoor work and apparently normal yielded 0.165 g. per litre, containing 0.004 g. N. Only a few allergic urines, however, have been examined quantitatively in this way, because Oriel and Barber have provided abundant data and also because the values so obtained are liable to certain errors. As will be shown the material consists largely of salts precipitated by the alcohol from the urine entangled in the ether, and some nitrogenous substances are similarly precipitated or adsorbed, which have no direct relation to the ether test. Further, only after being shaken several times with fresh quantities of ether does the urine cease to give the typical honeycomb layer. The vigour of the shaking also materially affects the degree of emulsification, and so the quantity of material obtained by a single treatment.

Ash content of the crude material.

The ash content of the precipitate (determined by ignition at a moderate temperature, moistening the ash with dilute sulphuric acid and then strong ignition) varied from 70 to 87 % of the material which had been dried at 100°. It consisted, as might be expected, of sodium sulphate with about 6 % of calcium sulphate and sometimes a little phosphate.

Fraction of the crude material soluble in cold water.

The greater part of the crude material was soluble in water and the solution obtained, using only a small excess of cold water above that required to dissolve the salts, gave only a moderate ether reaction. The p_H of this solution was about 3. The solution did not give the protein reactions distinctly but gave Molisch's reaction which was probably due to a trace of glucose in this

case. Evidently the material responsible for the ether reaction is sparingly soluble in concentrated salt solution at p_H 3.

Of the crude dry material from urine passed during a severe attack of asthma 89 % was soluble under these conditions. The crude material had 4.5 % N, of which 3.2 % was soluble, including 0.4 % of ammonia-N (these figures being all percentages of the crude material). The soluble portion contained practically all the inorganic matter and some organic matter. No appreciable precipitate was obtained on saturating the solution with ammonium sulphate and boiling. As the substance responsible for the ether reaction was almost entirely in the insoluble fraction the soluble material was not examined further.

The protein fraction.

The residue obtained on washing the crude material free from salts with cold water will be referred to as the "protein fraction" because it consisted almost entirely of protein or related substance. It was soluble to some extent in cold water, even after acidification, and could not therefore readily be obtained free from salts by shaking the ether with acidified water after removal of the urine. In its moist finely divided state it dissolved instantly in very dilute alkali to give an almost clear, slightly brown solution. When dried at 100° for some time it became insoluble in dilute ammonia. Addition of acetic acid to the filtered alkaline solution produced slight opalescence but no precipitate in the cold. On boiling the acidified solution a little coagulated protein was precipitated. When this had been removed the filtrate, although giving all the colour reactions of proteins, did not give a precipitate with potassium ferrocyanide. This applies both to material from asthmatic and that from non-asthmatic urine.

Half saturation of the acidified solution (freed from coagulable protein) with ammonium sulphate gave only a trace of precipitate in the cold even after 12 hours, but, on boiling, a flocculent precipitate was obtained and the filtrate from this, both in the case of asthmatic and normal urine was found to be free from protein. The precipitate was soluble in hot water and did not come down again on cooling.

When the acetic acid solution (freed from coagulable protein) was saturated completely with ammonium sulphate a precipitate was obtained in the cold. This was removed by centrifuging, dissolved in water, dialysed until free from sulphate and reprecipitated by addition of 3 vols. of alcohol. The precipitate was separated, washed with alcohol and ether and dried at 100°. It formed slightly pigmented transparent scales. It was acid to litmus and was readily soluble in water at p_H 8. It was precipitated by Esbach's reagent, but not by ferrocyanide. It contained no phosphorus, and had 12.5 % N (micro-Kjeldahl) on an ash-free basis. By an adaptation of Weiss's modification of Millon's reaction the tyrosine content of material from asthmatic urine was estimated to be about 2 %, and the tryptophan by the May and Rose colorimetric method as modified by Boyd [1929] was found to be 0.5 %. The

glyoxylic reaction was definite but faint. Cystine was present as shown by the lead acetate test. Molisch's test was positive. The low nitrogen content, together with the low tryptophan content, points to the presence of a large prosthetic group. This is confirmed by the behaviour of the material on heating with Fehling's solution, slow but distinct reduction taking place. A control test, using purified coagulated fish-muscle protein, showed no reduction under the same circumstances. It appears probable therefore that carbohydrate is present in combination with a protein.

The only difference observed hitherto between the material isolated from normal and that from asthmatic urines is one of quantity.

Determination of the protein nitrogen of urine.

If a sediment is present in the urine the sample is first made alkaline to dissolve uric acid and protein. 25 cc. of the filtered urine are acidified with acetic acid and 75 cc. of 93 % alcohol are added. After 12 hours the precipitate is separated on the centrifuge, dissolved in water, and the solution is dialysed in a thin collodion membrane for a week in presence of toluene, the water being frequently changed. The contents of the membrane are then transferred to an evaporating basin, evaporated to 5 cc. and transferred to a hard glass boiling-tube, and the nitrogen content is determined. Almost identical results may be obtained by direct dialysis of the urine without alcohol precipitation, but by the above method the urea, hippuric acid and other substances are eliminated first.

Protein nitrogen of normal and pathological urines.

A few normal and pathological urines have been examined by the above method, and the results are given in Table I. Further work is being carried out in this direction, but from the present results it is evident that there may be a normal urinary protein content even in the cachexia of cancer. The constancy of an increased protein excretion in allergic conditions in particular is being investigated.

Table I. *Protein (i.e. non-dialysable) nitrogen of some normal and pathological urines.*

Urine	Non-dialysable nitrogen (g. per litre)
1. Normal (apparently)	0.014
2. Normal (apparently)	0.024
3. Cancer (extreme cachexia)	0.011
4. Cancer	0.016
5. Cerebral tumour	0.009
6. Besnier Prurigo	0.053
7. Asthma (in attack)	0.030
8. Cow (healthy)	0.020

Discussion of the nature of the protein.

Considerable confusion exists as regards protein substances in urine. The term "proteose" has often been employed loosely. From the description of the product isolated from normal and asthmatic urines it appears to be a mixture of heat-coagulable protein and a mucoid. The practical differentiation between mucoids and glucoproteoses is not clear. Phosphorus was not found in the purified material, *i.e.* after precipitation and dialysis, but the crude protein fraction from one asthmatic urine contained 0.3 % of phosphorus. It appears that little or no nucleoprotein is present.

Mörner [1895] made a detailed study of protein substances in normal urine. He found a substance which he called "urinary mucoid" identical with the well-known "nubecula" of cooled urine and obtained by him by allowing the filtered fresh urine to stand in presence of chloroform. Its composition and reactions corresponded closely with those described above for the substance isolated by means of the ether reaction, after removal of irreversibly heat-coagulable protein. He also found that normal urine contains a little serum-albumin, which, on addition of acetic acid to urine and shaking with chloroform, is precipitated chiefly in combination with chondroitinsulphuric acid, but also in combination with glycuronic acid and nucleic acid. The properties of the mucoid and of the loose compound of serum-albumin with chondroitinsulphuric acid, as found by Mörner, were very similar, but the mucoid was not precipitated on shaking its solution with organic solvents in presence of chondroitinsulphuric acid (which is said to be present in all urines), although it was so precipitated in the absence of that substance. On this his separation of two different mucin-like substances depended. A consideration of the precipitation reactions of these bodies and of the phenomenon of the precipitation of proteins on the interfaces between solvents and water throws doubt on Mörner's conclusion that the mucoid was not precipitated from urine by shaking with solvents.

In order to settle this point 100 cc. of fresh normal urine were acidified with sulphuric acid and shaken repeatedly with fresh quantities of ether until a honeycomb layer was no longer obtained. The aqueous layer was then treated with 3 vols. of 93 % alcohol and after some hours the precipitate was removed on the centrifuge, dissolved in water, and the solution dialysed for a week in presence of toluene, the water being frequently changed. The non-dialysable nitrogen was then determined. The protein nitrogen of the urine was similarly determined omitting the treatment with ether. It was found that whereas the urine contained 0.014 g. of protein nitrogen per litre, the protein nitrogen left after repeated treatment with ether was 0.0026 g. per litre. Mörner repeatedly found mucoid corresponding to about 0.003 g. of nitrogen per litre, but it is probable that the precipitation was by no means complete. Further, it is not to be expected that shaking with ether can effect complete separation of the protein. It seems likely that the 0.0026 g. per litre of residual

non-dialysable nitrogen was due partly at least to a residue of the same kind of protein as that precipitated. These considerations support the view that Mörner was mistaken in supposing that he had effected a separation of two different kinds of mucin-like substances. Both, if present, are precipitated on shaking the acidified urine with solvents.

The irreversibly-coagulable protein (from asthmatic urine) was boiled repeatedly with water faintly acidified with acetic acid, allowed to stand in water at p_H 8 for 15 hours, washed repeatedly with distilled water by decantation, and then with alcohol and ether and dried at 100° . Its nitrogen content, allowing for 1.3 % of ash present, was 14.7 %. The alkaline treatment was intended to remove loosely combined precipitants such as chondroitinsulphuric acid, mentioned by Mörner. As the nitrogen content of serum-albumin is 15.9 % it is possible that the coagulated material was serum-albumin contaminated with mucoid (which, according to Mörner, is itself a protein precipitant) or with chondroitinsulphuric acid.

It is hoped by means of biological tests to establish the identity or non-identity of the proteins of normal and allergic urines with components of blood-serum and to confirm Oriel and Barber's observations on the specific antigenic properties of the urinary protein of allergic conditions, using purified material.

CONCLUSION.

The substances in normal and asthmatic urines responsible for the ether reaction and isolated thereby are an irreversibly coagulable protein, possibly identical with serum-albumin, and a mucoid containing 12.5 % of nitrogen, possibly derived from the urinary passages.

The author acknowledges the receipt of a personal grant from the Asthma Research Council. He is indebted to Dr W. Robson for advice and to Drs G. H. Oriel and G. W. Bray for pathological material.

REFERENCES.

- Boyd (1929). *Biochem. J.* **23**, 78.
- Mörner (1895). *Skand. Arch. Physiol.* **6**, 332.
- Oriel and Barber (1930). *Lancet*, 231.
- Ploz (1890). *Jahresber. Thier. Chem.* **20**, 215.
- Smith (1892). *Lancet*, 688.

XCII. NOTE ON THE REACTIVATION OF REDUCTASE IN WASHED YEAST PREPARATIONS.

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(Received April 28th, 1931.)

It was shown by Harden and Norris [1914, 1915] that after being washed with water dried (Lebedev) yeast did not reduce methylene blue; this property however was restored on the addition of boiled washings and also of definite chemical substances such as salicylaldehyde, benzaldehyde and lactate. It was suggested by Lebedev and Griaznov [1912] that a co-reductase was necessary for the action of reductase, in a manner analogous to that of co-enzyme; this theory, though later withdrawn by Lebedev [1927] is upheld by Euler and his colleagues [Euler and Nilsson, 1925] who regard the co-reductase as being identical with co-mutase and the co-enzyme of alcoholic fermentation. In considering the evidence for this view the possibility must be borne in mind that boiled yeast extract may restore the activity of the reductase only by reason of hydrogen donors contained therein rather than by the presence of a true co-enzyme. Euler and Nilsson [1927] however showed that neither a co-enzyme preparation which had been considerably purified nor a hexosediphosphate diminished the time required for the reduction of methylene blue by washed dried yeast when added separately, whereas when both were present together decoloration took place rapidly. If however the addition of co-enzyme together with hexosediphosphate, aldehyde or other substance induces alcoholic fermentation in a washed preparation, the chemical changes involved may provide an easily oxidisable substance which will allow the reduction of methylene blue to proceed, without any necessity for postulating a co-reductase. Consequently, it is unjustifiable to draw any conclusions as to the necessity for a co-reductase from experiments on the reactivation of reductase in washed yeast by the addition of any substance, if the possibility exists that such a reactivation is a secondary effect of alcoholic fermentation induced by the substance.

The earlier experiments of Harden and Norris were made at a time when failure to ferment sugar in presence of inorganic phosphate was accepted as evidence of the complete absence of co-enzyme from the washed yeast preparation. Meyerhof [1918] showed that the presence of a small amount of hexosediphosphate rendered this test more delicate, since its fermentation required a smaller concentration of co-enzyme. Later Kluyver and Struyk

[1927, 1928] showed that the reactivation of apozymase by acetaldehyde described by Harden [1917] did not take place in maceration residue after adequate washing; while Stheeman [1930] has made it clear that four distinct stages of inactivation of the fermenting complex of zymin can be attained by various degrees of washing. In the first stage the washed zymin is reactivated by a hydrogen acceptor, such as methylene blue or acetaldehyde; in the second by hexosediphosphate; in the third by hexosediphosphate and a hydrogen acceptor, and in the last by hexosediphosphate and co-enzyme. It has been confirmed in this laboratory that the adequate criterion for absence of co-enzyme in a washed preparation is a failure to ferment in presence of hexosediphosphate, and that the reactivation by acetaldehyde previously described [Harden, 1917] does not take place in the complete absence of co-enzyme.

It is obvious that a preparation in any of the first three stages of inactivation of zymase may be reactivated by aldehydes and hexosediphosphate with regard to reductase, if the fermentation so induced does in fact provide a suitable hydrogen acceptor. It seemed probable that the results obtained by Harden and Norris [1914] might require revision in the light of the later evidence as to the stages of inactivation by washing. It was therefore decided to repeat their experiments on the reactivation of reductase by chemical substances, using a washed preparation whose state of inactivation was checked by simultaneous fermentation experiments. Since it was impossible to use the same type of yeast as before, dried baker's yeast was chiefly used, as this is easily freed from co-enzyme. The results given below were obtained from various washed preparations of dried yeast.

Preparation 1. Dried baker's yeast washed three times by centrifuging with 8 parts of water, once on the filter with 5 parts, and dried with acetone and ether.

Preparation 2. Same dried yeast, washed four times with 8 parts of water by centrifuging, once on the filter and suspended in water (5 parts).

Preparation 3. Same dried yeast, washed five times with 8 parts of water, once on the filter with 5 parts and suspended in water (5 parts).

Preparation 4. Dried Copenhagen yeast, washed five times with 8 parts of water by centrifuging, once on the filter and suspended in water (5 parts).

Fermentation experiments. 0.2 g. of the washed preparation, 0.5 cc. of 20 % fructose and 0.1 cc. 0.6 M K_2HPO_4 were placed in each of three micro-fermentation flasks to which were added:

Flask (a) 0.5 cc. 0.1 M hexosediphosphate;

(b) 1.0 cc. yeast extract (prepared from ale yeast zymin);

(c) 0.5 cc. hexosediphosphate + 1.0 cc. yeast extract;

together with water to make a total volume of 2.5 cc. The flasks were placed in a thermostat at 30°, the CO_2 evolved being measured (Table I).

Reductase experiments. The reducing action of the preparations towards

methylene blue was then tested in the presence of various substances. A measured quantity of the washed suspension with 0.1 cc. of 1/1000 methylene blue and 0.1 cc. toluene was incubated at 26° in a total volume of 4 cc. with the addition of the desired substance. After constant observation for some hours, the mixtures were allowed to stand overnight, the minimum reduction time being given for such as were decolorised in the morning (Table II).

Table I. *Fermentation by washed yeast preparations. cc. CO₂.*

Time in hrs.	Washed preparation							
	1		2		3		4	
	2	20	2	20	2	20	2	20
Flask (a)	0	>5	0	>5	0	0.15	0	0.25
(b)	0.06	>5	0	>5	0	0	0.06	1.45
(c)	1.49	>5	3.16	>5	2.6	>5	0.24	2.20

Table II. *Reduction time of methylene blue by washed preparations.*

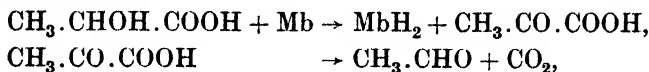
Additions	Preparation			
	1	2	3	4
(a) —	>5 hrs.	>4 hrs.	>4 hrs.	>3 hrs.
(b) 0.5 cc. hexosediphosphate	>5 hrs.	3½ hrs.	3 hrs.	>3 hrs.
(c) 1 cc. yeast extract	>5 hrs.	15 min.	—	5 min.
(d) 1 cc. yeast extract + 0.5 cc. hexosediphosphate	20 min.	15 min.	—	5 min.
(e) 0.01 cc. salicylaldehyde	>5 hrs.	>4 hrs.	>4 hrs.	>3 hrs.
(f) 0.01 cc. benzaldehyde	>5 hrs.	>4 hrs.	>4 hrs.	>3 hrs.
(g) 0.1 cc. 0.01 M lactate	1 hr.	30 min.	20 min.	15 min.
(h) 0.1 cc. lactate + 0.5 cc. hexosediphosphate	45 min.	—	20 min.	—
(i) 0.1 cc. 0.01 M succinate	—	3 hrs.	—	—

Another sample of washed yeast did not reduce methylene blue after 20 hours alone, but did so in 30 minutes with yeast extract, 1½ hours with lactate and 2½ hours with methylglyoxal.

The washed yeast preparations varied somewhat in their properties according to the manner of preparation. Thus preparation 1, which had been treated with acetone, required both yeast extract and hexosediphosphate for a rapid reduction, while preparation 2 reduced rapidly with yeast extract alone, although there was no significant difference in their fermenting power. The Copenhagen yeast reduced quickly with yeast extract, but fermented very poorly. The preparations were all consistent in that in the absence of yeast extract and hexosediphosphate, neither fermentation nor reduction took place during the period of constant observation (2–6 hours), though in the majority of cases both fermentation and reduction took place overnight. In all cases the addition of lactate caused a reduction of methylene blue in a time comparable with that taken by an addition of yeast extract + hexosediphosphate. With preparation 3, for instance, where no fermentation occurred in absence of co-enzyme, lactate effected reduction in 20 minutes, and with preparation 5, which did not reduce methylene blue alone, in 1½ hours. Succinate and methylglyoxal were also capable of acting as hydrogen donors,

though less rapidly than lactate but salicylaldehyde and benzaldehyde were ineffective.

During the reduction of methylene blue by washed dried yeast in presence of lactate, the lactate is decomposed with evolution of CO_2 , presumably according to the equations



the pyruvic acid being decomposed by the carboxylase of the yeast. The acetaldehyde produced might conceivably also have acted as an acceptor for the dehydrogenation of lactate. That this was not the case was shown in the following experiments.

0.2 g. washed yeast + 0.25 cc. K_2HPO_4 were placed in each of three micro-fermentation flasks to which were added

1. 1 cc. 1 % lactate + 0.01 g. methylene blue + 1.25 cc. H_2O ;
2. 1 cc. lactate + 0.03 g. methylene blue + 1.25 cc. H_2O ;
3. 1 cc. lactate + 0.1 cc. 10 % acetaldehyde + 1.24 cc. H_2O .

The flasks were placed in a thermostat at 30° and the CO_2 measured. Flask 1 became decolorised in 45 minutes, giving 0.73 cc. CO_2 ; flask 2 in 80 minutes, giving 1.82 cc. CO_2 , the theoretical amounts for these quantities of methylene blue being 0.8 and 2.4 cc. respectively. No evolution of CO_2 took place in flask 3. Rather less than the equivalent amount of CO_2 was evolved for the methylene blue reduced, showing that the acetaldehyde produced in the reaction or added independently did not act as a hydrogen acceptor for lactate. Moreover, the addition of pyruvic acid, which might liberate acetaldehyde in a more reactive state, was also ineffective in inducing a fermentation of lactate.

SUMMARY.

The addition of lactate, succinate or methylglyoxal restored the power of reducing methylene blue to yeast preparations washed to a point at which no fermentation of hexosediphosphate occurred. From these results it is concluded that a co-enzyme is probably not required for the action of the reductase in yeast preparations.

REFERENCES.

- Euler and Nilsson (1925). *Z. physiol. Chem.* **149**, 44.
 ——— (1927). *Z. physiol. Chem.* **162**, 84.
 Harden (1917). *Biochem. J.* **11**, 64.
 ——— and Norris (1914). *Biochem. J.* **8**, 100.
 ——— (1915). *Biochem. J.* **9**, 330.
 Kluyver and Struyk (1927). *Proc. K. Akad. Wetensch. Amsterdam*, **30**, 569.
 ——— (1928). *Biochem. Z.* **201**, 212.
 Lebedev (1927). *Z. physiol. Chem.* **172**, 255.
 ——— and Griaznov (1912). *Ber. deutsch. chem. Ges.* **45**, 3256.
 Meyerhof (1918). *Z. physiol. Chem.* **101**, 165.
 Stheeman (1930). *Proc. K. Akad. Wetensch. Amsterdam*, **33**, 889.

XCIII. FERMENTATION BY YEAST PREPARATIONS.

- I. THE EFFECT OF MONO-iodoacetate ON THE FERMENTATION OF HEXOSEDIPHOSPHATE.**
- II. THE ACTION OF ARSENATE ON THE INDUCTION PERIOD OF ZYMIN.**

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(Received April 20th, 1931.)

I. THE EFFECT OF MONO-iodoacetate ON THE FERMENTATION OF HEXOSEDIPHOSPHATE.

It has been shown [Macfarlane, 1930] that a yeast hexosephosphatase incapable of esterifying and fermenting sugar can be obtained, whose action in liberating inorganic phosphate from hexosediphosphate is accelerated by arsenate only in the presence of the co-enzyme of alcoholic fermentation and with an equivalent production of CO_2 . Fluoride, which inhibits the hydrolysis either in presence or absence of arsenate only by about 10 %, decreases the fermentation of hexosediphosphate in presence of arsenate by 80 %.

According to Lohmann [1930] both muscle extract and yeast maceration juice in the presence of fluoride convert glycogen or starch or added hexosediphosphate into a diphosphoric ester much more resistant to acid hydrolysis than the Harden and Young ester. Lipmann [1930] and Lundsgaard [1930, 1] have shown that the poisoning of muscle by sodium fluoride and by mono-iodoacetate produces similar effects, *i.e.* the muscle can contract without formation of lactic acid but with the formation of a hexosediphosphoric ester, probably of the same type as Lohmann's ester. Moreover mono-iodoacetate prevents the esterification of sugar and phosphate by yeast maceration juice [Lundsgaard, 1930, 2]. The action of this salt was also investigated by Yamazaki [1930], who pointed out that its inhibitory effect on the fermentation of hexosediphosphate by maceration juice was greater in the presence of arsenate, but came to the conclusion that the main effect was an inhibition of the esterifying power with phosphate. It seemed of interest to determine the effect of mono-iodoacetate on fermentation of hexosediphosphate by the hexosephosphatase referred to above, so that the process might be uncomplicated by the presence of zymase.

A hexosephosphatase was prepared by the autolysis of dried baker's yeast,

as previously described in detail [Macfarlane, 1930]. The preparation contained no co-enzyme and did not esterify or ferment sugar with added co-enzyme. A typical experiment was carried out as follows. Exp. 1. 1 cc. of a 20 % solution of the enzyme, 2 cc. 0.1 *M* potassium hexosediphosphate and 1 cc. boiled yeast extract were placed in each of four test-tubes, to which were added:

- (a) 2 cc. H_2O ;
- (b) 0.6 cc. 1 % potassium mono-iodoacetate + 1.4 cc. H_2O ;
- (c) 0.3 cc. 0.1 *M* Na_2HAsO_4 + 1.7 cc. H_2O ;
- (d) 0.3 cc. 0.1 *M* Na_2HAsO_4 + 0.6 cc. potassium mono-iodoacetate
+ 1.1 cc. H_2O .

The tubes were placed in a thermostat at 30°, 1 cc. samples being withdrawn at intervals and the inorganic P estimated by the Briggs method in the trichloroacetic acid filtrates. The quantities of phosphate liberated in 30 minutes, as mg. P per cc., were in (a) 0.175; in (b) 0.160; in (c) 0.984; and in (d) 0.551. The effect is more strikingly shown by plotting the rates of liberation of inorganic P (corrected for the change occurring in the absence of co-enzyme) over short time intervals, as in Exp. 2 (Fig. 1). The curve (b) obtained

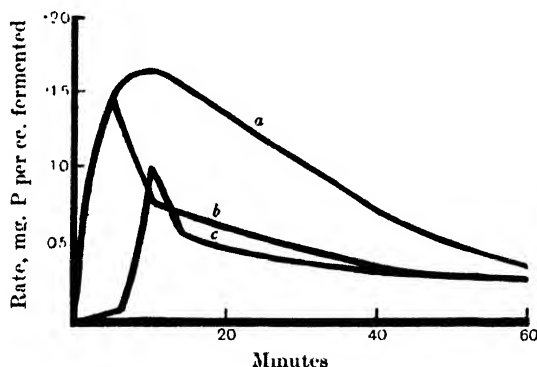


Fig. 1. Fermentation of hexosediphosphate.

- Exp. 2. (a) 2 cc. 10 % hexosephosphatase + 5 cc. 0.1 *M* hexosephosphate + 2 cc. yeast extract + 6 cc. 0.1 *M* As_2O_5 + 2.4 H_2O .
 (b) 2 cc. 10 % hexosephosphatase + 5 cc. 0.1 *M* hexosephosphate + 2 cc. yeast extract + 6 cc. 0.1 *M* As_2O_5 + 1.4 cc. 1 % mono-iodoacetate + 1 cc. H_2O .
 (c) 2 cc. 10 % hexosephosphatase + 5 cc. 0.1 *M* hexosephosphate + 2 cc. yeast extract + 6 cc. 0.1 *M* As_2O_5 + 0.6 cc. 1 *M* NaF + 1.8 cc. H_2O .

with mono-iodoacetate is almost identical with (a), the control with arsenate, for the first 5 minutes and then rapidly falls to a low rate coinciding with that reached by the curve (c) representing a similar fermentation in presence of fluoride. The decrease in rate shown in curve (a) after 20 minutes is due to the decreasing concentration of hexosediphosphate, 50 % of which has been decomposed at this point.

The nature of the ester remaining in the present case has not been ascertained but in view of the similarities already noticed between fluoride and mono-iodoacetate it appears probable that the reason for the inhibition of

fermentation lies not so much in a direct effect on the enzyme as in the fact that it is now acting on a less congenial substrate.

The effect of potassium mono-iodoacetate on esterification and fermentation by yeast juice.

Unlike maceration juice, which was used by Lundsgaard, yeast juice contains a fair amount of glycogen and a distinction has therefore to be drawn between the esterification of added sugar and that of the glycogen already present in the medium.

Autofermentation of yeast juice. Flasks containing the desired mixtures were placed in a thermostat at 30°, the CO₂ evolved being measured. Samples for phosphate estimation were taken at zero time and subsequently at intervals. After fermentation had been allowed to proceed for an hour, arsenate was added to make a final concentration of 0.01 *M*. The course of the esterification and fermentation is illustrated in Table I and Fig. 2.

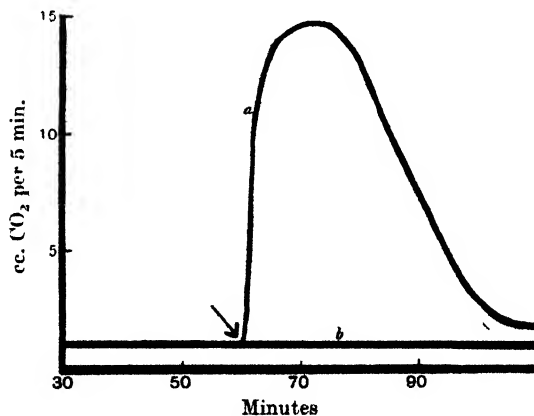


Fig. 2. Autofermentation of yeast juice.

(a) Control. (b) + mono-iodoacetate. The arrow marks addition of 3 cc. 0.1 *M* arsenate.

Table I.

(a) 20 cc. yeast juice + 3 cc. 0.6 *M* K₂HPO₄ + 3 cc. H₂O.

(b) 20 cc. yeast juice + 3 cc. 0.6 *M* K₂HPO₄ + 3 cc. 2 % mono-iodoacetate.

	(a)	(b)
	mg. P esterified	mg. P esterified
1st hr.	+41.5	+33.6
2nd hr.	-89.6	+12.5
(with arsenate)		
	CO ₂ as mg. P (CO ₂ ≡ P)	CO ₂ as mg. P (CO ₂ ≡ P)
1st hr.	21.4	20.7
2nd hr.	91.5	10.6

The autofermentation of the juice is considerable, but is slower than the fermentation of sugar and is not appreciably diminished by mono-iodoacetate. A slow esterification of phosphate takes place, which is more than equivalent to the CO₂ produced; in presence of 0.1 *M* iodoacetate 80 % of this esterification still occurs. On adding arsenate the rate of CO₂ evolution in the normal juice

increases considerably for a time and then returns nearly to its original level the excess CO_2 being almost exactly equivalent to the amount of esterified P which disappears during this period. This confirms Meyerhof's [1927] statement that the accelerated rate in presence of arsenate in glycogen fermentation is due entirely to the increased rate of fermentation of accumulated hexosediphosphate and not to an acceleration of the diastatic activity of the juice.

In the presence of mono-iodoacetate no acceleration takes place with arsenate, although the concentration of esterified P is of the same order as that in the control juice at this point; the esterification of phosphate however continues, 12.5 mg. being esterified in the next hour. The iodoacetate has therefore inhibited the acceleration by arsenate without greatly affecting the esterifying power with regard to glycogen.

Fermentation of sugar by yeast juice. On adding phosphate and mono-iodoacetate simultaneously to yeast juice fermenting sugar, an immediate esterification of phosphate occurs, accompanied by an equivalent evolution of CO_2 , 90 % esterification taking place as compared with the control juice. The basal rate of fermentation, however, quickly falls and is not accelerated by the addition of arsenate, while on subsequently adding phosphate no rapid esterification and fermentation take place. A slow esterification of phosphate continues still, corresponding approximately to the rate of glycogen esterification. The esterification of sugar by yeast juice is therefore inhibited by a concentration of mono-iodoacetate which only slightly affects that of glycogen.

The course of a typical experiment is shown in Table II.

Table II.

(a) 20 cc. yeast juice + 3 cc. 0.6 M K_2HPO_4 + 2 g. fructose + 3 cc. H_2O .				
(b) 20 cc. yeast juice + 3 cc. 0.6 M K_2HPO_4 + 2 g. fructose + 3 cc. 2 % mono-iodoacetate.				
	(a)		(b)	
	mg. P esterified	CO_2 as mg. P ($\text{CO}_2 \equiv \text{P}$)	mg. P esterified	CO_2 as mg. P ($\text{CO}_2 \equiv \text{P}$)
30 min.	+ 47.4	45.0	+ 43.3	20.7
1 hr. 10 min. (with arsenate and phosphate)	+ 3.0	555.0	+ 15.2	32.0

The salt acts in a manner similar to fluoride, though its effect is not so immediately apparent. The direct fermentation of hexosediphosphate either by yeast juice or by the hexosephosphatase preparation and co-enzyme is also inhibited as shown by the failure to respond to the presence of arsenate by an increased rate.

The results quoted above afford additional confirmation of the theory of Meyerhof [1927] that a direct fermentation of hexosediphosphate takes place in fermenting yeast preparations and in muscle extracts, in addition to the co-enzyme-free hydrolysis described by Harden and Young [1910]. The possibility is not excluded that a labile sugar may be formed by hydrolysis of

hexosediphosphate in presence of co-enzyme, capable of fermentation without previous re-esterification, rather than a disruption of the molecule with the simultaneous production of phosphate and some intermediate compound, *e.g.* methylglyoxal; moreover, although in the experiments here recorded with the hexosephosphatase preparation the accelerated decomposition of the ester in presence of arsenate was always accompanied by evolution of CO_2 , it is possible that this may not hold for other yeast preparations.

II. THE ACTION OF ARSENATE ON THE INDUCTION PERIOD OF ZYMIN.

It has been shown [Harden, 1925; Harden and Macfarlane, 1928] that mixtures of zymín and sugar solution normally show an induction period varying in duration with the volume of solution added before the onset of rapid fermentation occurs, and that arsenate increases the length of this period during which no evolution of CO_2 takes place. It was noticed that the inorganic phosphate present in trichloroacetic acid filtrates from the mixtures increases during the induction period. If this phosphate arises from a fermentation of hexosediphosphate, the rate of liberation should increase on addition of arsenate and simultaneously, since no CO_2 is evolved, an accumulation of the hexose residue, either unchanged or in the form of an intermediate product, should occur. If, on the other hand, the phosphate is not increased by the addition of arsenate, it must be derived either from a hydrolysis of hexosediphosphate or from some other change in the phosphorus compounds present.

Duplicate series of flasks were prepared containing 2 g. of zymín and known volumes of sugar solution or water; to one arsenate was added in the desired concentration (0.02–0.002 *M*), the corresponding flask of equal volume acting as the control. In some cases hexosediphosphate was added to shorten

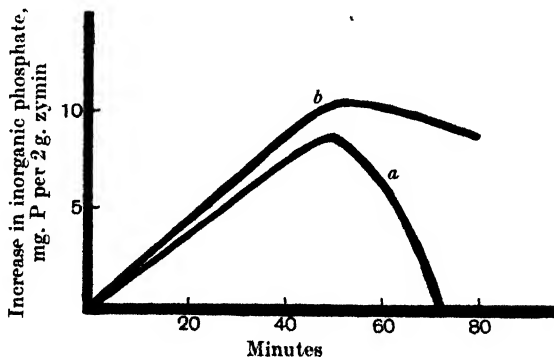


Fig. 3. Induction period of zymín fermentation.

(a) 2 g. zymín + 40 cc. H_2O . Induction time, 80 min.

(b) 2 g. zymín + 8 cc. 0.1 *M* Na_2HAsO_4 + 32 H_2O . Induction time, ∞ .

the induction period. The flasks were placed in a thermostat at 30° and the inorganic phosphate was estimated by the Briggs method in the trichloroacetic acid filtrates of samples taken at frequent intervals. Figs. 3 and 4 illustrate typical experiments.

It was found that the maximum liberation of inorganic phosphate took place in the larger volumes employed, that is where the liberation of the available phosphate was completed before any esterification of phosphate prior to fermentation developed, the rate of liberation being linear and unaltered by the addition of arsenate. As the volume decreased, the initial rate of increase was still linear but first gradually and then abruptly decreased as esterification was established, the middle portion of the curve (Fig. 3 *a*) representing the balance between a constant liberation and an increasing esterification. The addition of arsenate did not increase the rate of liberation

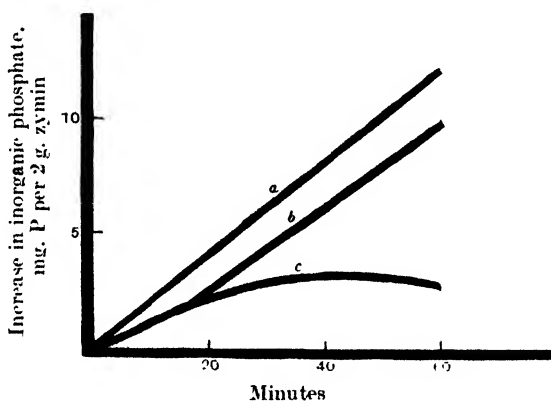


Fig. 4. Induction period of zymin fermentation.

- (*a*) 2 g. zymin + 1 g. fructose + 60 cc. H_2O . Induction time, > 80 min.
 (*b*) 2 g. zymin + 1 g. fructose + 2.4 cc. 0.5 M Na_2HAsO_4 + 2.5 cc. 0.1 M K hexosediphosphate + 55.1 H_2O . Induction time, $> 3\frac{1}{2}$ hr.
 (*c*) 2 g. zymin + 1 g. fructose + 2.5 cc. hexosediphosphate + 57.5 H_2O . Induction time, 0.

(Fig. 4. *a* and *b*), though by its effect in delaying esterification and thus maintaining the original linear rate, the total amount of the increase in inorganic phosphate was greater than in the control in those cases where the normal induction time was shorter than the time of complete hydrolysis. Estimation of methylglyoxal by distillation and precipitation as the *p*-nitrophenylosazone showed that no accumulation of triose had taken place during the induction period.

It was also found that the total amount of acid-soluble phosphorus present in the trichloroacetic acid filtrate rose during the induction period by an amount approximately equal to the increase in inorganic phosphate. Thus in one case the inorganic phosphate increased from 9.13 to 19.4 mg. during an induction period of 60 minutes, while the total acid-soluble P increased from 23.2 to 32.0 mg. It seems probable that the increase in inorganic phosphate must be due to changes in some other phosphorus-containing substance in zymin during the induction period rather than to the decomposition of hexosephosphoric esters. It must at least be concluded that the action of arsenate in prolonging the induction is not directly due to its specific action in accelerating the rate of fermentation of hexosediphosphate.

It is interesting to note that the same treatment which decreases the rate of fermentation of yeast and enables it to acquire the power of responding to phosphate, such as drying or treating with acetone, also injures the indophenol oxidase-cytochrome system [Keilin, 1929], one of the instances of injury being that cytochrome in dried yeast and zymin is reduced in amount and undergoes oxidation and reduction more slowly than in untreated yeast. In yeast suspensions warmed to 52° the reduction of cytochrome is markedly accelerated by lactate and also by succinate [Keilin, 1929], while arsenate increases the reduction time in suspensions of fresh yeast [Bierich and Rosenbohn, 1929]. The antagonistic action of these two salts in this case is parallel with their effect on the induction period of zymin fermentation, which is decreased and prolonged by lactate and arsenate respectively [Harden and Macfarlane, 1928]. This indicates that the phenomenon may be associated with the cytochrome content of the yeast preparation, and attempts are being made to obtain experimental evidence on this question.

SUMMARY.

1. The fermentation of hexosediphosphate by the hexosephosphatase preparation previously described in presence of co-enzyme and arsenate is inhibited by mono-iodoacetate.
2. The esterification of sugar by yeast juice is inhibited by mono-iodoacetate in concentrations which only slightly inhibit that of glycogen.
3. Arsenate does not accelerate the rate of fermentation by yeast juice in presence of mono-iodoacetate.
4. The rate of liberation of inorganic phosphate during the induction period of zymin fermentation is not accelerated by arsenate, showing that this phosphate does not arise by fermentation of hexosediphosphate.

I wish to record here my gratitude to Prof. A. Harden for his constant help and advice throughout this work.

REFERENCES.

- Bierich and Rosenbohn (1929). *Z. physiol. Chem.* **184**, 246.
Harden (1925). *Biochem. J.* **19**, 477.
— and Macfarlane (1928). *Biochem. J.* **22**, 786.
— and Young (1910). *Proc. Roy. Soc. Lond. B* **82**, 321.
Keilin (1929). *Proc. Roy. Soc. Lond. B* **104**, 206.
Lipmann (1930). *Biochem. Z.* **227**, 110.
Lohmann (1930). *Biochem. Z.* **222**, 325.
Lundsgaard (1930, 1). *Biochem. Z.* **217**, 162.
— (1930, 2). *Biochem. Z.* **220**, 1.
Macfarlane (1930). *Biochem. J.* **24**, 1051.
Meyerhof (1927). *Biochem. Z.* **183**, 178.
Yamazaki (1930). *Biochem. Z.* **228**, 123.

XCIV. THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE PRECIPITATING AND PROTECTIVE ACTIONS OF PROTEINS ON COLLOIDAL GOLD AND GUM BENZOIN.

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IN a previous communication [Wright and Kermack, 1923, 1], evidence has been adduced that the power of gelatin to protect colloidal gum benzoïn against precipitation by sodium chloride depends on the p_H and is minimal at the isoelectric point. The variation of protection with p_H was small and so it appeared desirable to confirm the results and to extend the observations to other proteins and to another lyophobic colloid. The present communication deals primarily with the protective action of gelatin, ovalbumin, serum-albumin, the serum-globulins, oxyhaemoglobin and edestin on colloidal gum benzoïn and colloidal gold at various hydrogen ion concentrations. The effect of variation of p_H on the protective power of colloids is of interest not only from the theoretical point of view, but also because such variations may be of importance in biological systems and in technical processes.

The opportunity was taken to make further observations on the action of the proteins on the lyophobic colloids in the absence of salt. It was shown by Wright and Kermack [1923, 1, 2] that colloidal gum benzoïn was precipitated by very small concentrations of gelatin, serum-globulin and oxyhaemoglobin at reactions on the acid side of the isoelectric point, and by somewhat higher concentrations at reactions approaching or corresponding with the isoelectric point [see also Kermack and McCallum, 1924]. Michaelis and Nakashima [1923] almost simultaneously, working with mastic sol, developed a method for the determination of the isoelectric point of proteins in which use was made of the same principle, and in this way obtained values for the isoelectric points of the serum-albumins of various animals. Reinders and Bendien [1925, 1928] have also investigated the reaction between lyophilic and lyophobic colloids at various reactions, and these authors observed that colloidal gold was coagulated by small concentrations of gelatin on the acid side of the isoelectric point. Similar observations were made with other proteins, caseinogen and sodium lysalbuminate. They observed that at p_H 6

or higher all the proteins used exerted no precipitating but only protective action, but the precise effect of variation of p_H on the latter does not appear to have been closely investigated. Especially in view of the proposal of Michaelis and Nakashima to use the phenomenon as a method for the determination of isoelectric points, it seemed desirable to enquire more closely into its general application to various proteins and lyophobic colloids.

EXPERIMENTAL.

The colloidal gum benzoin was prepared as described by Wright and Kermack [1923, 1, p. 637]. and the colloidal gold by adding to 100 cc. of distilled water 1 cc. of 1 % solution of photographic gold chloride, 1 cc. of a 1.4 % solution of potassium carbonate (previously dried and ignited), heating to boiling point, and then adding with vigorous shaking, after removal from the flame, 1 cc. of a solution of neutralised 0.40 % formaldehyde.

To each of a series of tubes were added 0.25 cc. of protein of appropriate concentration, 0.5 cc. of acid or alkali or buffer mixture as the case may be, and 1.0 cc. of gold or benzoin sol. After mixing, the tubes were allowed to stand overnight, when, if desired, readings were made in order to record the interaction between the protein and sol in the absence of salt. 0.25 cc. of sodium chloride (10 %) was now added and the tubes shaken and read after a further 24 hours' standing. The readings were recorded in tables similar to those given in previous papers [Wright and Kermack, 1923, 1, 2], and from these readings figures were prepared showing the regions in which complete or almost complete precipitation takes place. To save space, only the figures are given. In the case of the gold, in certain tubes the precipitate was red or purple, and not blue. The regions in which red precipitates were obtained are shown by shading. All the protein concentrations given refer to the final concentrations. As the readings in the absence of salt were made when the volume was 7/8 of the volume after the addition of the salt, the concentrations of protein should really be 8/7 of those given, but as the protein concentrations were doubled in consecutive tubes, the correction required is insignificant.

In the case of colloidal gum benzoin, which itself has a p_H of about 3.8, the various organic acids present in low concentration have a sufficient buffering effect to stabilise the p_H over the range employed in these experiments. In the case of colloidal gold, however, it was usually necessary to employ small concentrations of buffer mixtures in order to obtain satisfactory results. The concentration of buffers was small ($N/200$) in comparison with that of the sodium chloride employed to effect the precipitation, so that any effect of the ions of the buffer in sensitising or protecting the gold would be small in comparison with the effect of the sodium and chlorine ions. The exact buffers used are stated in the figures. The p_H measurements were usually made colorimetrically by means of the B.D.H. capillator, and in some cases confirmed by means of the quinhydrone electrode.

It is to be emphasised that the transition from complete protection to complete precipitation is usually a gradual one, and that, further, in this transition region the exact amount of precipitation depends on the precise conditions of the experiment. The aim of these experiments was, therefore, to determine broadly the approximate conditions under which precipitation and protection take place, and the figures should be interpreted accordingly.

Gelatin. Isoelectric gelatin was prepared according to the method of Loeb [1922, p. 35], and the protein content determined by drying to constant weight at 100° . Dilutions in distilled water were prepared and the experiment carried out as described above. The results are shown in Figs. 1 A and 1 B.

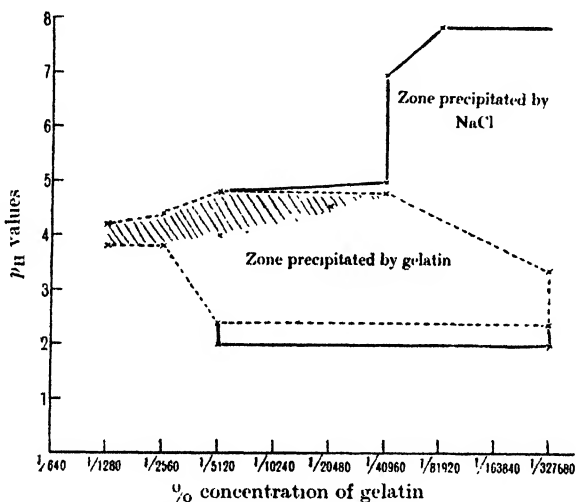


Fig. 1 A. Action of gelatin on gold sol. Final concentration of buffer used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of gelatin $p_H 4.7$.

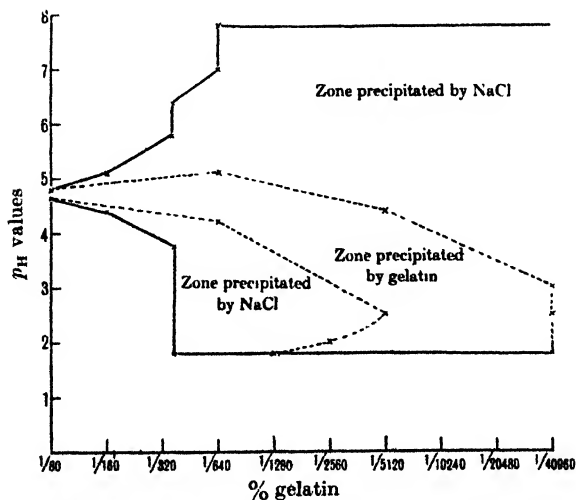


Fig. 1 B. Action of gelatin on gum benzoin. No buffers used.

Ovalbumin. Crystalline ovalbumin (Merck), 1 g. in 100 cc. of distilled water, was filtered to remove a small amount of flocculent insoluble material, and the requisite dilutions in distilled water prepared. The results are shown in Figs. 2 A and 2 B.

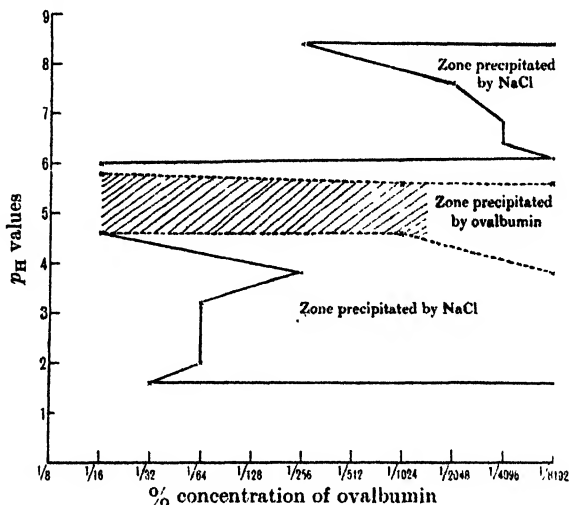


Fig. 2 A. Action of ovalbumin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of ovalbumin p_H 4.6-4.8.

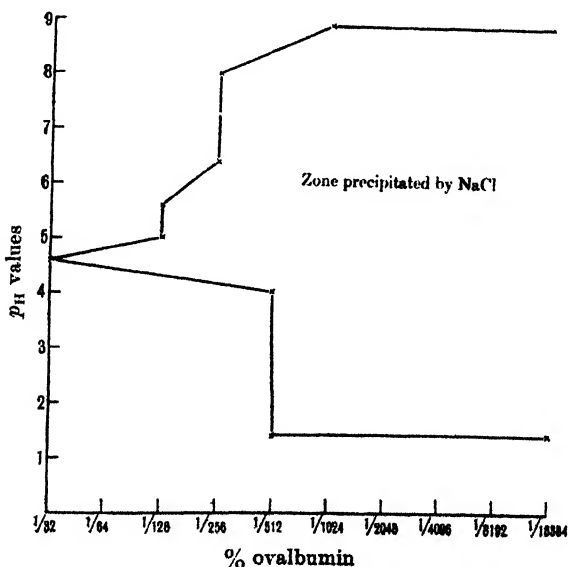


Fig. 2 B. Action of ovalbumin on gum benzoin. No buffers used.

Serum-albumin, pseudoglobulin and euglobulin. A quantity of human plasma, separated from oxalated blood, was fractionated by ammonium sulphate precipitations. The fibrinogen was removed by quarter saturation and

the serum-globulins in the filtrate precipitated by half saturation, filtered and washed with half-saturated ammonium sulphate. The albumin was precipitated by full saturation. Each fraction was dissolved in distilled water, reprecipitated, washed, redissolved in water and dialysed through a collodion membrane until practically free from sulphate. The euglobulin, which had precipitated during dialysis, was separated from the pseudoglobulin and was dissolved in 0.75 % sodium chloride solution. The protein present in each solution was determined by means of the Folin-Wu method as in the determination of serum-proteins.

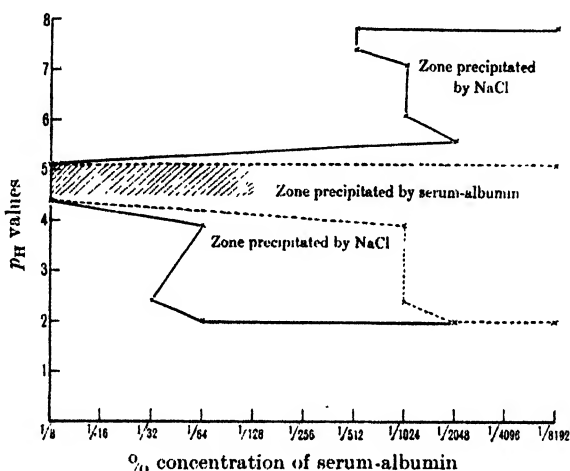


Fig. 3 A. Action of serum-albumin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of serum-albumin p_H 4.7-4.9.

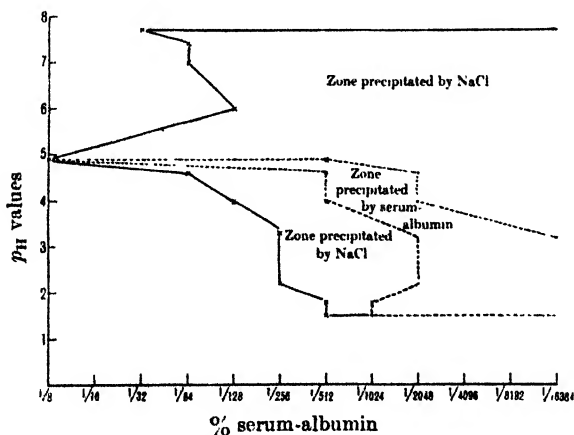


Fig. 3 B. Action of serum-albumin on gum benzoin. No buffers used.

The results appear in Figs. 3 A and 3 B (serum-albumin), 4 (serum-pseudoglobulin) and 5 (serum-euglobulin). In the case of euglobulin the original solution necessarily contained sodium chloride required to effect solution,

but the dilutions were made with distilled water so that in the lower concentrations of protein the concentrations of salt were correspondingly reduced.

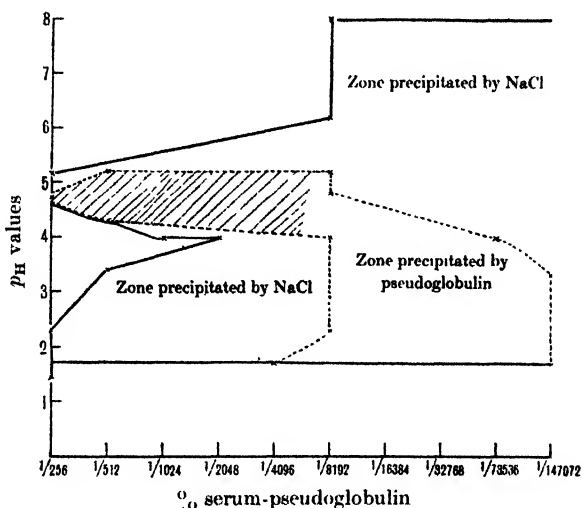


Fig. 4. Action of serum-pseudoglobulin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of serum-pseudoglobulin p_H 4.7–4.8.

In any case the final concentration of salt added was considerably greater than that present in the most concentrated protein solution used, so that,

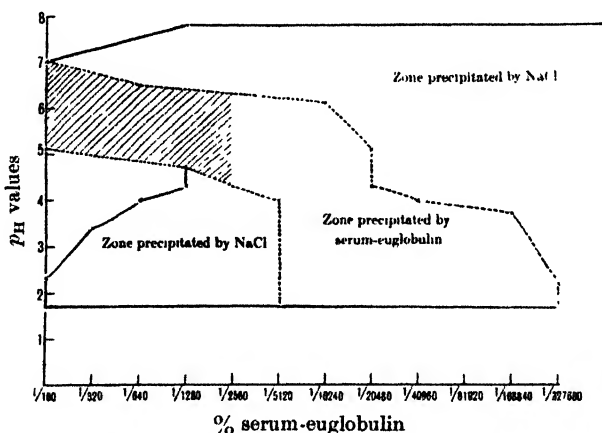


Fig. 5. Action of serum-euglobulin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of serum-euglobulin p_H 5.5.

although the concentration of salt in the final mixture was not constant, the variations were relatively small.

Oxyhaemoglobin. Human red blood corpuscles, centrifuged from oxalated plasma were washed several times with 0.9 % salt solution, mixed with distilled water to cause haemolysis and repeatedly centrifuged to remove stromata. The concentration of oxyhaemoglobin in the dialysed solution was

determined by using the Zeiss strophotometer to ascertain the extinction coefficient, the filter S 51 ($\mu\mu$ 490–530) being employed. The concentration of haemoglobin in a particular solution was found by the Kjeldahl method (total nitrogen), and from the extinction coefficient of this solution it was calculated that a 3 cm. layer of a 0.01 % solution had an absorption coefficient of 0.1358. The percentage concentration of any solution is therefore given by the formula $x = \frac{0.01\sigma}{0.1358}$, where σ is the observed extinction coefficient for a 3 cm. layer.

Dilutions in distilled water were prepared and the experiments carried out in the usual manner. Owing to the colour of oxyhaemoglobin it was difficult or impossible to make readings of the precipitation of gold in the ordinary way. It was found, however, that by observing the tubes in a beam from an arc lamp, the presence of particles of colloidal gold throughout the solution could readily be detected by the Tyndall effect. Tubes in which sedimentation of gold had not occurred gave a marked scattering of incident light. In the experiments with gum benzoin this difficulty did not arise, and readings could be easily made in the ordinary way. The results are given in Figs. 6 A and 6 B. It was found that in the tubes in which the p_H was 5 or

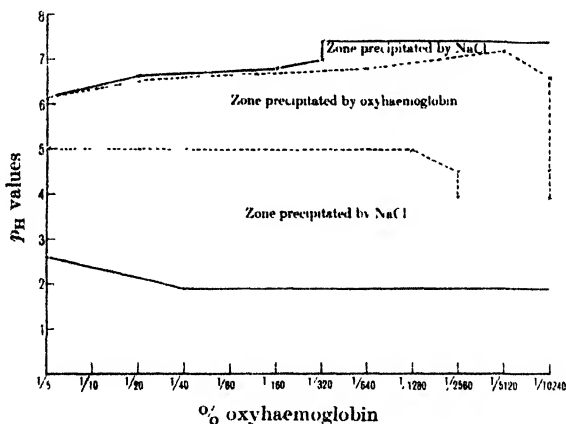


Fig. 6 A. Action of oxyhaemoglobin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of oxyhaemoglobin p_H 6.7–6.8.

less, a brown colour developed, clearly due to the splitting of the oxyhaemoglobin into haematin and globin. The results in this region should therefore be interpreted with caution.

Edestin. Several attempts were made to carry out experiments with edestin from hemp seed (Merck), but no satisfactory results could be obtained, owing to the very small solubility of this protein in distilled water in the neighbourhood of the isoelectric point. In the most concentrated solutions prepared no protection of gold or benzoin was observed between p_H 3.5 and 7.5. Above and below this range protection was observed with 0.05 % edestin. In another experiment in which, in order to obtain higher concentrations of edestin, the

protein was dissolved in 5 % sodium chloride and the solution adjusted to various reactions from p_H 3.0 to 8.5 by the addition of a suitable acetate-phosphate buffer mixture ($M/50$), no protection was observed when an equal volume of gold or benzoin sol was added to these mixtures. The final concentration of edestin in these experiments ranged from 0.1 to 0.0002 %, that of

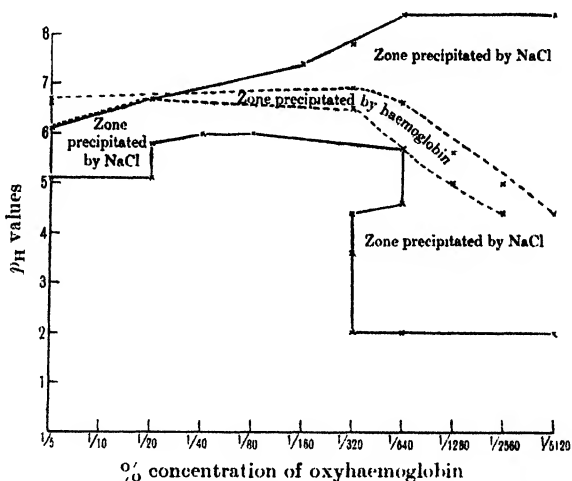


Fig. 6 B. Action of oxyhaemoglobin on gum benzoin. No buffers used.

salt being constant throughout. The gold in all the tubes was completely precipitated, but, in the more concentrated solutions and the more alkaline of these, the precipitates were distinctly red and graded steadily to blue, being quite blue in the dilute solutions (all reactions) and in acid solutions (all concentrations).

DISCUSSION.

As the results obtained with oxyhaemoglobin are in some respects anomalous, these will be discussed separately after dealing with the other proteins.

Precipitation in the absence of added electrolytes (excluding the experiments with oxyhaemoglobin). The observations are in agreement with those previously made by Wright and Kermack [1923, 1] and Michaelis and Nakashima [1923]. In the presence of small quantities of proteins on the acid side of the isoelectric point, and therefore positively charged, the discharge of the negative colloid takes place and precipitation occurs. When the concentration of protein is very low, precipitation is observed only at reactions well on the acid side of the isoelectric point, as otherwise the protein adsorbed on the negative colloid bears an insufficient number of positive charges to discharge the colloid. As the concentration of protein is increased the p_H at which precipitation occurs approaches more nearly to the isoelectric point. It is not to be expected that the limit p_H at which precipitation occurs will always coincide exactly with the isoelectric point, as it depends for example on such

factors as the degree of adsorption of the protein on the particles, the rate at which ionisation increases as the isoelectric point is left, and the protective power of the protein. If this latter were sufficiently great, it might prevent precipitation at a concentration of protein insufficient to bring the zone of precipitation up to the isoelectric point. There is also the possibility that the isoelectric point of the protein might be altered by adsorption, for example as the result of denaturation. Further, if we are to compare the isoelectric point of the protein with the limiting p_H at which precipitation occurs when the concentration of protein is increased, it is necessary to define the latter unambiguously. In the case of gelatin and mastic, or gelatin and gum benzoin, there is no ambiguity, as when the concentration of protein is increased the zone of precipitation narrows down and converges on a particular point which is not, however, necessarily the maximum value of p_H at which precipitation occurs. With lower concentrations of protein precipitation may occur at a higher p_H , although the mean p_H of the zone obtained at any particular protein concentration does not exceed, but tends to approach, the limiting p_H (see Fig. 1 B). In the case of a protein which, in the neighbourhood of the isoelectric point, brings about precipitation, even at high concentrations, it is clear that the mean p_H of the zone must be taken. The actual width of the zone itself at a given protein concentration depends on various factors, *e.g.* the concentration of buffer used, or the stability of the lyophobic colloid, but the mean p_H is relatively constant. We shall therefore consider the "limit mean" p_H of the zone in relation to the isoelectric point. It appears from the diagrams that when colloidal gum benzoin is used, this "limit mean" p_H does in fact approximately agree with the isoelectric point, but when gold is employed the agreement is not so good, and in the case of oxyhaemoglobin there is a discrepancy of more than one unit of p_H .

Another point which should be noted in connection with the precipitation of gold is that the precipitates were not infrequently red or purple in colour instead of blue. As the blue colour of gold precipitated by inorganic salts may be taken as an indication of the adhesion of the gold particles together, it would seem that the purple or red colour of these precipitates indicates that the gold particles were still virtually separate. The red precipitate showed itself chiefly in the immediate neighbourhood of the isoelectric point and in the presence of moderate concentrations of protein. It would appear that the protein micelles become aggregated round the gold particles and, as a result of this, they sediment relatively more easily. When the isoelectric point is reached and the charge on the protein virtually disappears, these aggregates, each containing a gold particle, may sediment, even although the pure protein is quite stable under these conditions. The gold particles will, however, be carried down in a dispersed condition, and a red precipitate will be formed. The zone of red precipitation was especially wide in the case of serum-euglobulin, and this is in agreement with the fact that this protein is relatively easily precipitated from solution.

Protection in the presence of electrolytes (excluding the experiments with oxyhaemoglobin). On the addition of salt (sodium chloride) any particles of the lyophobic colloid which have adsorbed no protein or only very small amounts will tend to lose their charges and will then be free to aggregate, and in the case of gold the colour will change to blue. Salt, in the concentration employed in these experiments, will have comparatively little direct effect on the proteins. It is not to be expected that the red region of precipitation will be markedly extended, and, further, the entanglement of the particles in the precipitated protein where this has occurred will prevent the change of colour to blue. The charge of the particles which are surrounded with protein will depend primarily on the relation of the p_H of the medium to the isoelectric point of the protein. Disregarding for the moment the tubes in which red precipitation occurred before the addition of salt, it appears in general that the region of blue precipitation occurs in more and more concentrated protein as the isoelectric point is approached. To this extent there is evidence that the protective action of the protein decreases in the neighbourhood of the isoelectric point. We may as a working hypothesis assume that the amount of adsorption of the protein is, at a first approximation, independent of the hydrogen ion concentration in the neighbourhood of the isoelectric point, and that an approximately similar film is formed whatever the reaction. At some distance from the isoelectric point, the protective effect of this film is reinforced by the charge which it carries. As the isoelectric point is approached this charge decreases, and so the apparent protective power is less. At the isoelectric point itself the charge is zero just as it was before the addition of salt, so that no change in the stability is effected by the addition of salt at this point. This accords with experiment except in the case of haemoglobin.

In the neighbourhood of the isoelectric point, approximately the same results are obtained with benzoin as with gold, except that in this case the precipitates are white in every instance, and there is no apparent difference corresponding to that of red and blue precipitates with gold. Protection is minimal at the isoelectric point and increases on each side. At some distance on each side gum benzoin differs rather remarkably from gold in one respect. With gold the maximum amount of protection on the alkaline side is much greater than on the acid side; with gum benzoin, on the other hand, approximate equality on each side was observed. Thus, for example, with serum-albumin at p_H 6.0 to 6.1 protection was observed at dilutions of 1/512 % and 1/64 % with gold and gum benzoin respectively, whereas at p_H 2.4 the corresponding dilutions were 1/16 % and 1/128 % respectively. It appears from these results that at p_H 2.4 eight times as much serum-albumin is required to protect gold as is required to protect gum benzoin, whereas at p_H 6.1 only one-eighth as much is required. It is rather difficult for any satisfactory explanation to be advanced, but it would appear that in acid solution there is relatively little adsorption of the positively charged protein ions by the gold.

Precipitation and protection by oxyhaemoglobin. The reaction between oxyhaemoglobin and gum benzoin in the absence of salt has already been described by Wright and Kermack [1923, 1], who used sheep's oxyhaemoglobin, and these results have been confirmed using human oxyhaemoglobin, the "limit mean" p_H found in this case being about 6.6. After the addition of sodium chloride the absence of protection was observed between p_H 5.4 and 6.0. The zone of minimum protection is thus distinctly to the acid side of the isoelectric point, which is about 6.8 [Michaelis and Airila, 1921; Geiger, 1931]. A cataphoretic experiment in a U-tube with the oxyhaemoglobin employed in the precipitation experiments showed that it was in fact positively charged at p_H 6.3. It was very curious to find that, on the addition of salt, partial or complete re-dispersion occurred in certain tubes at p_H 6.0 to 6.6. The results in the case of gold were even more unexpected. In the absence of salt the "limit mean" p_H at which precipitation occurs is about p_H 5.8 or even lower. When very little buffer was present, precipitation extended from p_H 5.0 to 5.5 in the higher concentrations of oxyhaemoglobin, and from p_H 5.0 to 6.0 in the lower concentrations. In an experiment carried out with phosphate buffers added to control p_H a widening of this zone occurred, but in the higher concentrations of protein, no precipitation took place above p_H 6.2, although at lower concentrations precipitation was observed from p_H 4.6 to 7.2. It is clear that, in the case of gold, the centre of the zone of precipitation is well below the isoelectric point, and without added buffers the whole zone may be well below it. When salt is added, very little change occurs on the alkaline side of the zone, except a widening in very low concentrations, but on the acid side extensive precipitation occurs and there is practically no protection. It has, however, to be remembered that the zone in the absence of salt extends down to p_H 5.0, and that below this p_H dissociation of oxyhaemoglobin into its components takes place, so that too much significance should not be attached to these observations below p_H 5.0.

It is clear that with this protein distinct abnormalities occur; in particular, it sometimes behaves as though its isoelectric point were well below the generally accepted value. There is no doubt that the gold particles in presence of haemoglobin or other proteins are positively charged in moderately acid and negatively in moderately alkaline solution. In the case of proteins other than oxyhaemoglobin, the zone of precipitation coincides with a zero charge on the protein, so that, on the acid side of this zone the particles are positively charged and on the alkaline side negatively charged. When the zone of precipitation coincides with the isoelectric point, this is entirely reasonable. But when the zone of precipitation is on the acid side, it follows that between this zone and the isoelectric point the particles must be negatively charged whilst the protein itself is positively charged. An experiment in which an attempt was made, by means of the ultramicroscope, to observe the charge on the gold particles in the presence of oxyhaemoglobin at p_H 6.3, indicated that it was in fact negative, but as it was relatively small, it was

difficult to ascertain its sign with absolute certainty. As mentioned above, a cataphoretic experiment by the U-tube method on the oxyhaemoglobin alone, showed that this protein had a positive charge at this p_H . Reference to previous work [Wright and Kermack, 1923, 1, 2; Kermack and McCallum, 1924; Michaelis and Nakashima, 1923] makes it clear that a region in which the particles are negatively charged while the protein is positively charged normally exists when the concentration of protein is sufficiently low, for in this case the zone of precipitation lies well below the isoelectric point. The explanation in this instance is clearly that the protein, being present in low concentration, must be at a p_H well below the isoelectric point in order that it should be sufficiently positively charged to discharge the negatively charged lyophobic particles completely. It is possible, then, that the abnormalities observed with oxyhaemoglobin may be due to the small adsorption of this protein on the colloidal particles in the neighbourhood of the isoelectric point. If only small adsorption takes place, it is possible for the positively charged protein to exist in stable equilibrium with negatively charged particles.

An alternative explanation might be based on the observation that in certain cases proteins are denatured when adsorbed on a surface [Loeb, 1923] and that the isoelectric point of the denatured protein might differ from that of the native protein. It might be that the adsorbed oxyhaemoglobin is denatured and now possesses an isoelectric point about one unit of p_H below that of the native protein. This hypothesis, however, appears to be untenable, in the first place, because it does not explain the difference in the "limit mean" p_H values in the cases of gold and of benzoïn, and in the second place, as the result of experiments made to determine the isoelectric point of oxyhaemoglobin denatured by heating to 90° for 5 minutes. By means of ultra-microscopic observations it was found that the particles had a positive charge at p_H 6.0, and a barely detectable positive charge at p_H 6.4. At p_H 6.5 and at p_H 7.0 no charge was discernible, whilst at p_H 7.5 the particles bore a small but negative charge. Heat-denaturation is of course not necessarily the same as surface denaturation, but the above two considerations appear to render the explanation unlikely.

It may be noted that, when a low concentration of calcium chloride (0.05*N*) was used in place of the usual concentration of sodium chloride employed in the experiments with colloidal gold and oxyhaemoglobin, a somewhat different result was obtained. The effect of the addition of calcium chloride was to extend the zone of precipitation on the alkaline as well as on the acid side. This fits in with the view that the particles of gold covered with oxyhaemoglobin are negatively charged at p_H 6.0 or more, as in that case the bivalent calcium ions would in this region effect precipitation more readily than the univalent sodium ions.

General. These results confirm the previous findings reported by Wright and Kermack [1923, 1] and almost simultaneously by Michaelis and Nakashima [1923] to the effect that, when low concentrations of protein are employed,

a zone of precipitation occurs on the acid side of the isoelectric point, and that, as the concentration of protein is increased, this zone approaches the isoelectric point, but does not pass it. As mentioned in the introduction, Michaelis and Nakashima have gone so far as to suggest that these observations form the basis of a method for the determination of the isoelectric point of soluble proteins, and have applied it in the case of a number of serum-albumins. It is to be observed, however, that they had apparently tested out the principle only over a limited range, namely with gum mastic and gelatin, and it is doubtful, in view of the variability of the isoelectric point of gelatin, how far their result, namely p_H 4.65, can be considered as a decisive proof of the validity of the method. The results now obtained with oxyhaemoglobin and gold show that, at least when certain lyophobic colloids are used, quite erroneous results may be obtained. Thus, although there is little doubt as to the general nature of the phenomena observed, there does not seem to be sufficient basis for applying the principle without qualification, as suggested by Michaelis and Nakashima, to the quantitative determination of the isoelectric points of proteins.

SUMMARY.

1. Observations have been made on the precipitation of colloidal gold and gum benzoin by gelatin, ovalbumin, serum-albumin, pseudoglobulin, euglobulin and oxyhaemoglobin at different concentrations of protein and of hydrogen ions. The observations confirm and extend the results previously obtained. In very low concentrations of protein, precipitation occurs on the acid side of the isoelectric point, but as the concentration of protein increases the zone of precipitation approaches the isoelectric point, and when sufficient protein is present, the centre of the zone, except in the case of oxyhaemoglobin, is at a point not significantly different from the isoelectric point.

2. In the presence of 1.25 % sodium chloride, minimum protection by these proteins, except oxyhaemoglobin, is observed at a reaction not significantly different from the isoelectric point. In the case of gold the precipitates observed in the neighbourhood of the isoelectric point are usually red in colour.

3. With oxyhaemoglobin abnormal results were obtained in respect both of precipitation and protection. This protein behaved as if its isoelectric point lay below its real value of about p_H 6.8. The anomaly is more pronounced in the case of gold than of gum benzoin. The explanation does not appear to depend on a change in the isoelectric point of the protein following on denaturation as a result of adsorption on the lyophobic particles, but may be due to small adsorption of the protein in the neighbourhood of the isoelectric point.

4. The results with oxyhaemoglobin and gold suggest that the method proposed by Michaelis and Nakashima for the determination of the isoelectric point of proteins is not of general application, and, in particular, may give erroneous results when gold is employed.

REFERENCES.

- Geiger (1931). *Proc. Roy. Soc. Lond. B* **105**, 368.
 Kermack and McCallum (1924). *Proc. Roy. Soc. Edin.* **45**, 71.
 Loeb (1922). *Proteins and the theory of colloidal behaviour*. (McGraw-Hill Book Co., New York.)
 — — (1923). *J. Gen. Physiol.* **5**, 479.
 Michaelis and Airila (1921). *Biochem. Z.* **118**, 144.
 — — and Nakashima (1923). *Biochem. Z.* **143**, 484.
 Reinders and Bendien (1925). *Chem. Weekblad.* **22**, 481.
 — — (1928). *Rec. Trav. Chim.* **47**, 977.
 Wright and Kermack (1923, 1). *Biochem. J.* **17**, 635.
 — — (1923, 2). *Biochem. J.* **17**, 658.

XCV. STUDIES IN THE METABOLISM OF TISSUES GROWING *IN VITRO*.

IV. EFFECT OF FRUCTOSE, GALACTOSE AND XYLOSE UPON THE AMMONIA AND UREA PRODUCTION OF EMBRYO KIDNEY TISSUE.

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(Received April 30th, 1931.)

PREVIOUS work has shown that by using the tissue culture technique the production of ammonia and urea by growing and non-growing cultures can be estimated. By comparison with controls it has been found that the growing cultures produce considerable amounts of these substances whereas the non-growing ones do not [Holmes and Watchorn, 1927], and that the presence of glucose, while favouring the growth of the tissue, inhibits the production of ammonia and urea during this growth [Watchorn and Holmes, 1927]. It seemed that it might be of interest to compare the effect of other sugars with that of glucose, in order to determine whether the utilisation of a carbohydrate necessarily has this "protein sparing" effect, or whether it depends upon the type of carbohydrate utilised and thus upon the method of breakdown.

The use of the tissue culture method has much to recommend it for work of this type. In the first place, it is valuable to know that one is dealing with the metabolism of growth and activity, and not with autolytic processes, or with the metabolism of cells surviving under conditions which are likely to upset its normal balance. Even in tissue culture the conditions are unnatural, but they are not sufficiently so to prevent the continuation of growth in the embryonic tissue taken. By noting the effect upon this growth of various substances added to the medium and utilised by the cells, it is possible to judge whether the breakdown of these substances is favourable or unfavourable to the activity of the tissue. Thus, in the earlier work with glucose, we found that the presence of the sugar prevented the ammonia and urea formation which is otherwise characteristic of growth, and also that this did not inhibit the growth, but encouraged it.

EXPERIMENTAL.

As in previous work embryonic rat kidney tissue has been used, and the details of technique have been the same as those previously described [Holmes

and Watchorn, 1927, 1929; Watchorn and Holmes, 1927]. The cultures were made in pyrex flasks, and the conditions of culture of the growing and non-growing tissues were identical, except that the growing fragments were allowed to rest upon very fine wisps of cotton-wool, while in the non-growing preparations the fragments were floated in a shallow layer of the medium. It is known that fragments floating freely in a medium do not grow to any appreciable extent. When necessary the system of controls described in our first paper was used, but in the experiments dealing with ammonia and urea production it was usually sufficient to compare the growing tissues with the non-growing ones, as these latter never formed any ammonia or urea and therefore gave exactly the same result as the controls.

At least two kidneys were used in each flask, and in order to obtain large enough differences, it was necessary to use four when the utilisation of carbohydrate was estimated. As it was obviously important to use only kidneys taken from the same litter throughout each experiment, the number of preparations that could be set up each time was limited by the size of the litter. When the nitrogen metabolism of cultures with and without sugar was being compared, the media were both made from the same embryo extract and were equally diluted, in the one case with Ringer's solution containing the carbohydrate and in the other with plain Ringer's solution. 2 cc. of the medium were used in each flask, and the cultures were incubated for 2 days.

The solutions of carbohydrate were sterilised by passing through a filter candle. We are inclined to believe that the method of sterilisation of the carbohydrate is important, and that after sterilisation by steaming there is likely to be a bigger disappearance of a sugar in culture than when the unheated sugar is used. The large breakdown of glucose observed in our earlier work may have been due to the fact that the medium was sterilised by heat. This idea is born out to some extent by an experiment with adult rat kidney tissue, in which the disappearance of steamed and unsteamed glucose in the presence of the tissue was estimated. It was found that there was a larger disappearance of the sugar which had been steamed. The sugar may or may not have been broken down by the tissue. Two or three years ago, one of us (B.E.H.) attempted in collaboration with A. Pirie to repeat the work of Borsook and Wasteneys [1925] in which they showed that glucose in the presence of an amino-acid and phosphate could reduce methylene blue under anaerobic conditions. We found that we could not repeat this unless we used, as did Borsook and Wasteneys themselves, glucose which had been sterilised by heating. With fresh glucose no reduction of the methylene blue was obtained. In these tissue culture experiments, therefore, it seemed safer to avoid complications by using filtered instead of steamed solutions.

Other details of technique will be found in earlier papers.

Growth¹ of tissue.

In most series one or more small control dishes were included, containing small amounts of tissue growing in the same medium as that used in the experimental flasks. This tissue could be stained and used for microscopical purposes. We have not adopted any accurate method for estimating growth, but the presence of fructose, galactose, and even xylose seemed to have an unmistakably favourable effect upon the growth of the tissues. This was particularly noticeable when the growth without added sugar was poor. From this it seemed almost certain that each of these sugars was utilised by the tissue, and we found later that definite amounts of the carbohydrate disappeared during the course of the experiment.

Effect on production of ammonia and urea.

Fructose behaved like glucose in that it inhibited either partially or completely the production of ammonia and urea by growing tissue in spite of the increased growth promoted by the sugar. In the presence either of glucose [Watchorn and Holmes, 1927] or of fructose (Table I) there was sometimes an actual disappearance of the urea already present, and as this did not appear as extra ammonia it seems possible that it was used by the growing tissue.

Table I. *Effect of fructose upon ammonia and urea production.*

Exp. no.	Non-growing tissue		Growing tissue		Non-growing tissue + fructose		Growing tissue + fructose		Increase in $\text{NH}_3\text{-N}$ + urea-N		Effect of fructose
	$\text{NH}_3\text{-N}$ (mg.)	urea-N (mg.)	$\text{NH}_3\text{-N}$ (mg.)	urea-N (mg.)	$\text{NH}_3\text{-N}$ (mg.)	urea-N (mg.)	$\text{NH}_3\text{-N}$ (mg.)	urea-N (mg.)	Without fructose (mg.)	With fructose (mg.)	
80	0.034	0.051	*0.040 0.044	0.070 0.065	0.030	0.058	0.030 0.033	0.066 0.061	0.019 0.014	0.008 Nil	Inhibition
81	—	0.056	—	0.118 0.092	—	0.055	0.036	0.081	0.062 0.036	0.026	Partial inhibition
82	0.031	0.060	0.038	0.071	0.030	0.062	0.030	0.055	0.011	Nil	Inhibition
84	0.040	0.097	0.059 0.050	0.163 0.117	0.040	0.081	0.057 0.040	0.077 0.088	0.066 0.020	0.016 0.007	Partial inhibition
86	0.043	0.066	0.060	0.105 0.072	0.039	0.067	0.043	0.054 0.056	0.039 Nil	Nil Nil	Inhibition (possible urea utilisation)

* Where two sets of figures are given, these refer to two separate preparations in the same experimental series, the control therefore being the same for both.

Quite unlike the results obtained with fructose and xylose those with galactose were markedly irregular. This irregularity persisted, although the experiments were repeated a great many times. Sometimes the production of ammonia and urea was inhibited, sometimes it was unaffected or even slightly increased (the increase probably being due to the relatively greater growth). Perhaps there is more than one path of galactose breakdown possible in the cultures, and the effect upon the nitrogen metabolism depends upon the path taken. It is certain, however, that the presence of galactose could

¹ By "growth" we mean increase of area, which is due to wandering of cells as well as to actual cell division.

at times have an inhibitory effect upon the formation of ammonia and urea, and when concentrations of sugar as high as 0.2 % or more were used, this inhibition was likely to be found in the majority of cases. Some results are given in Table II.

Table II. *Effect of galactose upon ammonia and urea production.*

Exp. no.	Non-growing tissue		Growing tissue		Non-growing tissue + galactose		Growing tissue + galactose		Increase in $\text{NH}_3\text{-N} + \text{urea-N}$		Effect of galactose
	$\text{NH}_3\text{-N}$ (mg.)	urea-N (mg.)	$\text{NH}_3\text{-N}$ (mg.)	urea-N (mg.)	$\text{NH}_3\text{-N}$ (mg.)	urea-N (mg.)	$\text{NH}_3\text{-N}$ (mg.)	urea-N (mg.)	Without galactose (mg.)	With galactose (mg.)	
76	0.030	0.045	0.024	0.039	0.029	0.043	0.037	0.072	Nil	0.029	Increase
	0.030	0.045			0.026	0.045	0.025	0.036		Nil	No effect
79	0.032	0.063	0.055	0.073	0.032	0.063	0.039	0.073	0.010	0.010	No inhibition
			0.055	0.088			0.025	0.056	0.025	Nil	Inhibition
			0.045	0.074			0.037	0.056	0.011	Nil	Inhibition
90	0.057	0.090	0.089	0.136	0.062	0.116	0.062	0.118	0.046	Nil	Inhibition
91	—	0.063	—	0.084	—	0.085	—	0.112	Nil	0.027	Increase
93	0.074	0.135	0.098	0.172	0.055	0.103	0.095	0.115	0.037	0.052	Increase
98	0.029	0.083	0.041	0.099	0.029	0.083	0.036	0.090	0.016	0.007	Partial inhibition
Higher concentrations of galactose (approx. 0.2 %).											
118	—	0.108	—	0.123	—	0.108	—	0.103	0.015	Nil	Inhibition
								0.033		0.025	Small increase
119	—	0.088	—	0.136	—	0.097	—	0.090	0.048	Nil	Inhibition

In no case have we found any inhibition of ammonia or urea formation by xylose, although there seems to be very little doubt that the tissue can cause this sugar to disappear from the medium. Xylose therefore is an interesting case of a carbohydrate which is in some way utilised without affecting the nitrogen metabolism of the tissue as measured by the production of these substances (Table III).

Table III. *Effect of xylose (0.2 %) upon ammonia and urea production.*

Exp. no.	Non-growing tissue		Growing tissue		Non-growing tissue + xylose		Growing tissue + xylose		Increase in $\text{NH}_3\text{-N} + \text{urea-N}$		Effect of xylose
	$\text{NH}_3\text{-N}$ (mg.)	urea-N (mg.)	$\text{NH}_3\text{-N}$ (mg.)	urea-N (mg.)	$\text{NH}_3\text{-N}$ (mg.)	urea-N (mg.)	$\text{NH}_3\text{-N}$ (mg.)	urea-N (mg.)	Without xylose (mg.)	With xylose (mg.)	
99	0.032	0.091	0.049	0.119	0.040	0.097	0.052	0.121	0.028	0.024	No inhibition
							0.055	0.121		0.024	
101	0.026	0.056	0.030	0.078	0.036	0.066	0.040	0.100	0.022	0.034	Slight increase
			0.035	0.082					0.026		
102	0.024	0.048	0.031	0.067	0.030	0.063	0.045	0.079	0.019	0.016	No inhibition
120	—	0.070	—	0.113	—	0.080	—	0.129	0.043	0.049	No inhibition
121	—	0.102	—	0.135	—	0.083	—	0.114	0.033	0.041	No inhibition

Disappearance of the sugars from the medium.

Apart from the added sugar the medium contained very little reducing substance. This is to be expected, since it was made from chopped embryo tissue which had already been once washed with Ringer's solution, and was finally diluted with twice its volume of Ringer's solution. The amount of reducing substance was about 0.1 mg. to 0.15 mg. in each culture. This is

unimportant when compared with the amount of sugar added (2.0 mg. approximately in each culture). We did not find that it changed appreciably during the experiment, and in any case it was not sufficient to account for the changes observed. We therefore ignored the reducing substances already present, and estimated the added sugar as reducing substance. The small amount of protein present was removed with colloidal ferric hydroxide, and the sugar estimated by the method of Hagedorn and Jensen. In order to obtain larger percentage differences we have made the final concentration 0.1 % instead of the 0.2 % used in most of the other experiments.

There seemed to be very little doubt that all the sugars added, fructose, galactose and xylose, could be utilised by the tissue, and it will be seen that the non-growing generally used less than the growing tissue. The control was a culture which had been kept in the ice-chest throughout the experimental period (Tables IV, V, VI).

Table IV. *Utilisation of fructose (estimated as glucose).*

Exp. no.	Control at 0° (mg.)	Non-growing tissue (mg.)	Growing tissue (mg.)	Control - growing tissue (mg.)
114	1.61	(1) 1.25 (2) 1.23	(1) 1.23 (2) 1.20	(1) 0.38 (2) 0.41
110	1.38	1.22	1.10	0.28
109	3.52*	(1) 3.20	(1) 3.23	(1) 0.29

* Very little tissue used.

It is interesting to find a small but definite disappearance of fructose, as it has been suggested [Campbell and Markowitz, 1927] that fructose is not utilised except by the liver.

Table V. *Utilisation of galactose (estimated as glucose).*

Exp. no.	Control at 0° (mg.)	Non-growing tissue (mg.)	Growing tissue (mg.)	Control - growing tissue (mg.)
115	1.39	1.02	(1) 1.06 (2) 0.93	(1) 0.33 (2) 0.46
116	1.35	0.99	0.67	0.68*

* Grew for 3 days.

It has been shown that galactose can give rise to lactic acid [Wierzechowski and Laniewski, 1931] and can raise the respiratory quotient and the heat production of dogs [Wierzechowski, 1931], while Sherif and E. G. Holmes [1930] have observed an increased oxygen uptake with mammalian nerve in the presence of galactose. It is perhaps worth noting that on one occasion the rise in blood- and urine-lactic acid found by Wierzechowski and Laniewski was much greater than in the other two experiments; some such irregularity may explain the lack of consistency in the effect of galactose on nitrogen metabolism in our experiments.

Table VI. *Utilisation of xylose (estimated as glucose).*

Exp. no.	Control at 0° (mg.)	Non-growing tissue (mg.)	Growing tissue (mg.)	Control - growing tissue (mg.)
113	2.03	—	1.70	0.33
106	1.81*	1.77	1.73	0.08
122	2.00†	1.60	1.46	0.54
108	1.99	1.77	1.64	0.35

* Very little tissue used.

† In this experiment the sugar was estimated by McCance's [1926] method for pentoses, and we are glad to have this opportunity of thanking him for help.

McCance and Madders [1930] showed that xylose was retained in the tissues after injection or absorption from the intestine, and Russell [1923] showed that pentoses were oxidised by tumour tissues, but that normal nerve tissues, including kidney, cannot oxidise them. The only exception was liver, which oxidised arabinose. He did not however use any embryonic tissue, and we think that the fact that we used embryonic tissue in an active condition influenced the results with xylose and with the other sugars.

SUMMARY.

1. The tissue culture method has been used to test on embryo kidney tissue the "protein sparing" action of fructose, galactose and xylose.

2. Each of these sugars tends to increase the growth of the tissue and each of them is utilised by the tissue, but while fructose constantly inhibits the production of ammonia and urea by the growing cells, galactose does so only irregularly and xylose not at all.

We are indebted to the Medical Research Council for personal grants.

REFERENCES.

- Borsook and Wastencys (1925). *Biochem. J.* **19**, 1128.
 Campbell and Markowitz (1927). *Amer. J. Physiol.* **80**, 548, 561.
 Holmes and Watchorn (1927). *Biochem. J.* **21**, 327.
 ——— (1929). *Biochem. J.* **23**, 199.
 McCance (1926). *Biochem. J.* **20**, 1111.
 ——— and Madders (1930). *Biochem. J.* **24**, 795.
 Russell (1923). *8th Sci. Rep. Imperial Cancer Res. Fund.* p. 11.
 Sherif and Holmes (1930). *Biochem. J.* **24**, 400.
 Watchorn and Holmes (1927). *Biochem. J.* **21**, 1391.
 Wierzechowski (1931). *Biochem. Z.* **230**, 187.
 ——— and Laniewski (1931). *Biochem. Z.* **230**, 173.

XCVI. THE EFFECT OF NARCOTICS ON SOME DEHYDROGENASES.

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(Received April 29th, 1931.)

ACCORDING to the well-known work of Warburg [1921] the inhibiting effect of narcotics on cell respiration, on fermentation by yeast and on oxidation of amino-acids on a charcoal surface is due to the preferential adsorption of these substances on the particular surfaces, and the reaction velocity is inhibited owing to the displacement of the reacting substances in question by the narcotics. That the narcotic action increases with the increase in the molecular weight in a homologous series, coupled with the fact that the surface activity of these substances increases also in the same order, is a strong support for the theory of Warburg. Nothing is known, however, about the particular constituents of the cell on which this displacement of adsorption takes place, though a good deal of emphasis has been laid on the rôle of lipoids in the phenomenon of narcosis [Loewe, 1913]. Henderson [1930] in his recent review of the theories of narcosis finds considerable difficulties in deciding on the substrate on which narcotics act in the known reversible manner. This question of the effect of narcotics on some particular constituents of the cells is naturally interesting and requires elucidation before the narcosis of cell respiration can be properly understood.

In a study of the cytochrome-indophenol oxidase system of cells, Keilin [1929] has put forward the view that cyanide inhibits the oxidation of cytochrome by the indophenol oxidase system. Narcotics, however, have little or no effect on the activity of the oxidase-cytochrome system. That is, they do not affect the oxidation of reduced cytochrome, but they inhibit the activity of dehydrogenases and hence the reduction of oxidised cytochrome. The inhibiting effect of narcotics on cell respiration is thus due to their inhibiting action on dehydrogenases in the cells.

The present paper contains a study of the effect of some narcotics on three dehydrogenase systems, namely xanthine-hypoxanthine dehydrogenase (xanthine oxidase), aldehyde dehydrogenase (Schardinger enzyme) and succinic dehydrogenase. Incidentally some experiments have been made on the question of the identity of xanthine oxidase with Schardinger enzyme.

EXPERIMENTAL.

The xanthine oxidase and Schardinger enzyme were prepared in the same sample in the form of the caseinogen preparation of Dixon and Thurlow

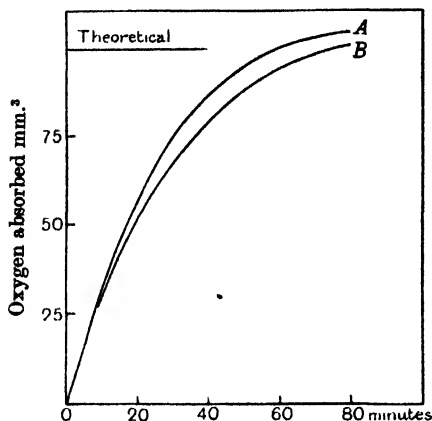


Fig. 1.

Fig. 1. Effect of ethylurethane on xanthine oxidase-hypoxanthine system.

A. Control.

B. Urethane 5 %.

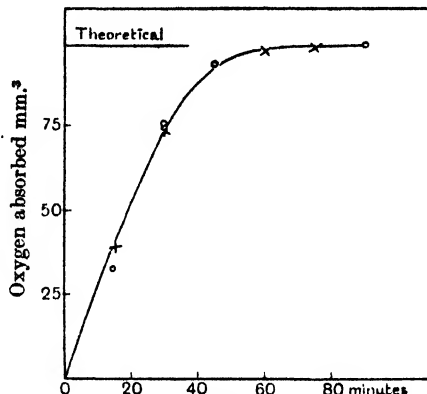


Fig. 2.

Fig. 2. Effect of phenylurethane on xanthine oxidase system.

○—○—○ Denotes control.

×—×—× Denotes urethane-saturated.

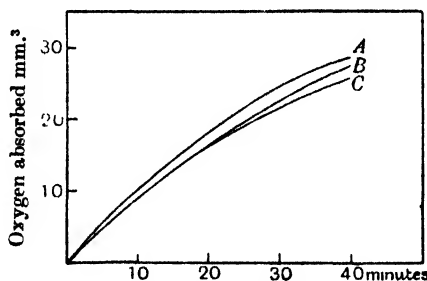


Fig. 3. Effect of narcotics on Schardinger enzyme.

A. Phenylurea 0.02 M.

B. Control and valeronitrile 0.11 M.

C. Ethylurethane 5 %.

The Barcroft cups in all these cases (Figs. 1, 2 and 3) were made up as follows: 0.5 cc. of 20 % caseinogen solution and 1.2 cc. phosphate buffer pH 7.6 were placed in both the cups. In the control, 0.3 cc. of 0.2 % hypoxanthine + 1 cc. of water was added in the right hand cup, whilst the left hand cup received 1.3 cc. water. The hypoxanthine was contained in a small Keilin tube which was tipped into the cup after temperature equilibration in the usual way and this was taken as the zero time. In the case of Schardinger enzyme, 0.5 cc. of 0.25 % piperonal was added in place of hypoxanthine, the quantity of water being reduced to 1.1 cc. When narcotics were added, the amount of water was reduced proportionately, the total volume being kept constant at 3 cc. in both the cups. The narcotic was added in both the cups, and in the case of phenylurethane an excess was present.

[1924, 1] from highly active milk. The succinic dehydrogenase was prepared from washed sheep's heart muscle according to the method of Keilin [1929].

The experiments were made both by the methylene blue technique (anaerobically) and by the oxygen-uptake method in a Barcroft micro-respirometer. The main results are summarised below. All the experiments were made at 37°.

(1) With xanthine oxidase using hypoxanthine as substrate, ethylurethane 5 %, phenylurethane, saturated solution, diethylurea 5 % and phenylurea 0.3 % had very little effect on the velocity of oxygen-uptake (Figs. 1 and 2).

(2) With Schardinger enzyme, using piperonal as substrate, ethylurethane 5 %, phenylurea 0.02 *M*, and valeronitrile 0.11 *M* had very little effect on the initial velocity of oxygen-uptake (Fig. 3).

(3) In the anaerobic reduction of methylene blue in presence of xanthine oxidase and Schardinger enzyme, similar results were obtained. The amount of methylene blue used was 1 cc. of 1:5000 solution in 5 cc. total volume.

(4) Narcotics had a strong inhibiting action on the succinic dehydrogenase system, the results of the oxygen-uptake and methylene blue reduction methods agreeing with each other. This action was found to be (in the case of ethylurethane which was the only narcotic examined) completely reversible. As the data are interesting, these results are given in some detail.

Effect of narcotics on succinic dehydrogenase.

The narcotics used in this investigation were ethylurethane, phenylurethane, diethylurea, phenylurea, propionitrile, valeronitrile and vanillin. The dehydrogenase was a washed muscle preparation containing approximately 100 mg. per 1 cc. of the suspension. The Barcroft cups were made up as follows: 0.5 cc. muscle preparation, 1 cc. phosphate buffer p_H 7.6, 1 cc. narcotic of known concentration. The right-hand cup contained in addition 0.5 cc. *M*/10 sodium succinate, whilst the same amount of water was added to the left-hand cup. In the control experiments, of course, no narcotic was added. The total volume

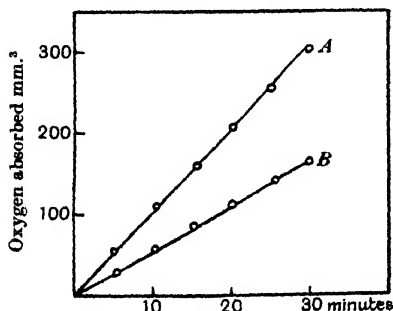


Fig. 4.

Fig. 4. Effect of diethylurea on the succinic dehydrogenase system.

A. Control.

B. Diethylurea 0.66 *M*.

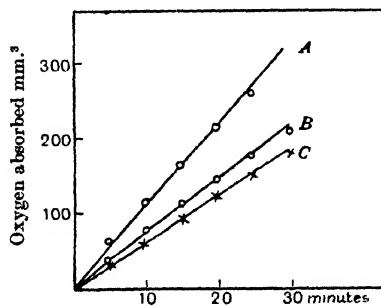


Fig. 5.

Fig. 5. Effect of phenylurea and vanillin on the succinic dehydrogenase system.

A. Control.

B. Phenylurea 0.02 *M*.

C. Vanillin 0.011 *M*.

in every cup was 3 cc. and the temperature of the bath 37° . In Figs. 4 to 7 the results are graphically shown. It will be observed that with all these narcotics the activity of the oxygen-absorbing system is greatly reduced.

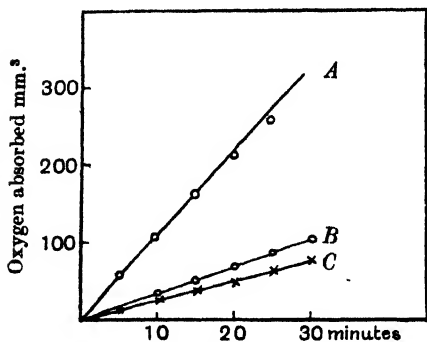


Fig. 6.

Fig. 6. Effect of nitriles on the succinic dehydrogenase system.
A. Control. B. Valeronitrile 0.11 *M*. C. Propionitrile 0.66 *M*.

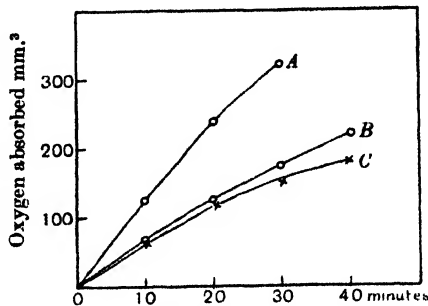


Fig. 7.

Fig. 7. Effect of urethanes on the succinic dehydrogenase system.
A. Control. B. Phenylurethane 0.003 *M*. C. Ethylurethane 0.66 *M*.

The majority of the narcotic concentrations were chosen in such a way (by preliminary tests) that the inhibition fell to about 50 % of the control. In other cases this value has been extrapolated. The following table summarises the results.

Table I.

Narcotic	Concentration of narcotic which inhibits about 50 % (<i>M</i>)		
	Oxygen-uptake in presence of succinic dehydrogenase	Respiration of nucleated erythrocytes— Warburg	Anaerobic reduction of methylene blue by succinic dehydrogenase
Ethylurethane	0.65	0.33	0.6
Phenylurethane	0.003	0.003	0.002
Diethylurea	0.35	0.52	0.2
Phenylurea	0.028	0.018	0.028
Propionitrile	0.48	0.36	—
Valeronitrile	0.08	0.06	—
Vanillin	0.011	0.02	0.022

The results obtained in this work are compared with those of Warburg on respiration by avian blood corpuscles. The last column contains the results obtained by the methylene blue technique. Since it will be interesting to compare the results of these methylene blue reduction experiments with the results obtained in oxygen absorption experiments, some of them are given here. It will be observed that the effects of narcotics in the case of the succinic dehydrogenase system are really on the hydrogen-activating mechanism of the system. Figs. 8–12 illustrate the main results obtained. Each Thunberg tube contained 1 cc. phosphate buffer of p_H 7.6, 0.5 cc. methylene blue 1:5000, 0.5 cc. muscle preparation, and 0.5 cc. sodium succinate *M*/10 unless

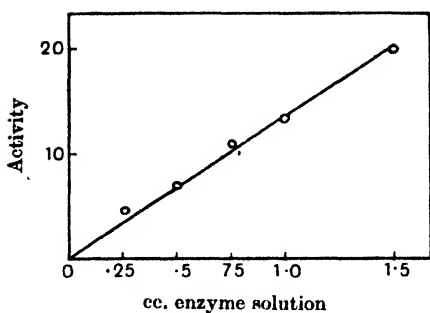


Fig. 8.

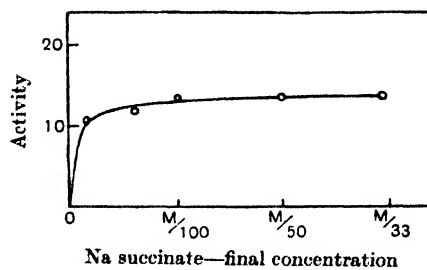


Fig. 9.

Fig. 8. Effect of varying enzyme concentration on the succinic dehydrogenase system.

Fig. 9. Effect of varying substrate concentration on the succinic dehydrogenase system.

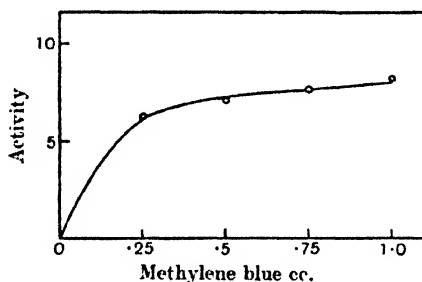


Fig. 10. Effect of varying methylene blue concentration on the succinic dehydrogenase system.

The curve shows the activity per unit quantity of methylene blue. The original concentration of methylene blue is 1 in 5000.

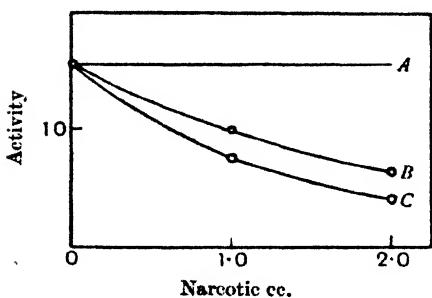


Fig. 11.

Fig. 11. Effect of urea on the succinic dehydrogenase system.

A. Activity without narcotic. B. Phenylurea 0.06 M solution.
C. Diethylurea 1.0 M solution.

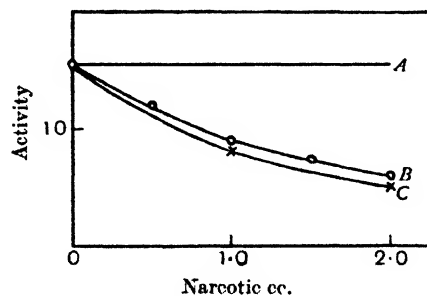


Fig. 12.

Fig. 12. Effect of urethanes on the succinic dehydrogenase system.

A. Activity without narcotic. B. Ethylurethane 17.8 %.
C. Phenylurethane 0.05 %.

otherwise stated, the total volume being 5 cc., made up either by addition of water or narcotic. The activity is the reciprocal of the time of reduction of methylene blue in minutes multiplied by 100. The data include the effect of varying enzyme concentration, the effect of different methylene blue concentrations, the effect of different substrate concentrations and the effect of different narcotics of different concentrations. The data given in the last column of Table I have been obtained from the experimental curves. It will be observed that the velocity of the reduction of methylene blue (activity) is directly proportional to the enzyme concentration, that the rate of methylene blue reduction at constant enzyme concentration depends on the concentration of methylene blue below a certain concentration, and that the rate of reduction of methylene blue is nearly constant within the range of substrate concentration studied in this paper [compare Ahlgren, 1926]. It will also be seen from Table I that in every case the higher homologue has a greater inhibiting (narcotic) action, a fact also observed by Batelli and Stern [1913, 1] but criticised by Vernon [1914], and the magnitudes of the individual values are also often not far from the values obtained by Warburg in his experiments on respiration by blood cells.

It will thus be observed from the above results that the muscle system containing succinic dehydrogenase and indophenol oxidase gives results in presence of narcotics entirely in accordance with the theory of Keilin. It is therefore interesting to enquire why narcotics fail to show any effect on xanthine oxidase or Schardinger enzyme. Curiously enough, both these systems are also not sensitive to cyanide, whereas oxidation of succinate in presence of the muscle system is sensitive to cyanide. This is no doubt due to the fact that cyanide inhibits the indophenol oxidase system which is absent from the xanthine or Schardinger enzyme. It is to be noted at this place that nitriles (propionitrile, valeronitrile) had no effect on the reduction of methylene blue by succinic dehydrogenase, though they inhibited the oxygen absorption. This is to be explained by the fact that the .CN group does not affect the hydrogen-activating system. Regarding the difference in the behaviour of these two types of dehydrogenases in presence of narcotics nothing definite can be said at present, but some related facts may be stated here. Batelli and Stern [1913, 2] showed that there is some connection between the ability of narcotics to coagulate nucleoproteins and to inhibit the action of succinic dehydrogenase. The muscle preparation used in this work is a coarse suspension which easily settles down in presence of narcotics. The caseinogen preparation of xanthine oxidase or Schardinger enzyme is, however, more soluble and gives a slightly opalescent solution which is not precipitated in presence of narcotics of the concentration used in this investigation. It was found, however, in several instances that by increasing the concentration of the narcotic to a high value (for instance, ethylurethane 14 %), some diminution in the velocity of oxygen-uptake in presence of xanthine oxidase could be observed. This effect may be due to a real coagula-

tion effect. As however such high concentrations are not to be considered as narcotic concentrations, these results were not followed up by any further work. Mention may also be made here of the interesting attempt of Bernard [1885], who tried to classify narcotisable and non-narcotisable ferments on the basis of their being associated either with living protoplasmic mass or with non-living and non-protoplasmic mass.

THE IDENTITY OF XANTHINE OXIDASE WITH SCHARDINGER ENZYME.

Warburg in his experiments used vanillin as a narcotic, and it has been found by me in this paper to have a narcotic action on succinic dehydrogenase. Since vanillin is an aldehyde, it could not be used as a narcotic as such with the caseinogen preparation of Dixon and Thurlow, as this preparation catalyses the oxidation of both hypoxanthine and aldehydes. Some preliminary experiments on the oxidation of hypoxanthine in presence of the caseinogen preparation, however, showed that the presence of vanillin affects the rate of hypoxanthine oxidation. It has previously been observed by Dixon and Thurlow [1924, 2] that uric acid inhibits the oxidation of aldehydes in presence of Schardinger enzyme and they advance this as a strong point in favour of the view that xanthine oxidase and Schardinger enzyme are identical. This view is also strongly supported by the high degree of specificity of the enzymes found by Coombs [1927]. The result obtained by me points to the fact that in presence of vanillin the two substrates are competing with each other, producing a competitive inhibition. The competition is so great that in experiments in which a saturated solution of vanillin was placed in both the cups of a Barcroft apparatus and hypoxanthine only in the right cup, no movement of the liquid in the manometer took place in half an hour. This showed that the oxygen-uptake in the two cups was balanced and the presence of a saturated solution of vanillin had completely excluded the hypoxanthine from the enzyme surface. Since Wieland and Macrae [1930] consider that they have observed a summation effect with two substrates (hypoxanthine and aldehyde), it was thought worth while to obtain some comparative data on this point. The experiments have shown that with hypoxanthine on one hand and vanillin (*m*-methoxy-*p*-hydroxybenzaldehyde) and piperonal (3:4-methylenedioxybenzaldehyde) on the other hand, no additive effect was observed. In every case the combined velocity of oxygen-uptake was midway between the velocities of oxygen-uptake of the separate systems. Figs. 13 and 14 show the results. The concentration of vanillin used was 0.5 % and of piperonal 0.25 %, other conditions being the same as described before. It will be seen that as the aldehyde concentration increases the total oxygen-uptake falls, though, had there been two different enzymes, the oxygen-uptake would have been greater than when hypoxanthine was present alone. The decrease in the oxygen-uptake is due to the fact that the aldehyde, which has a much lower rate of oxygen-uptake, displaces a portion of the hypoxanthine and hence lowers the rate of oxygen-uptake of the whole system. This result

points very strongly to the conclusion that the two dehydrogenases are identical.

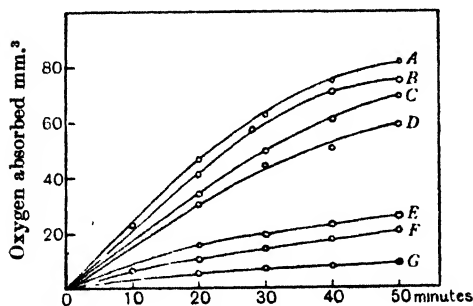


Fig. 13.

Fig. 13. Effect of vanillin on hypoxanthine oxidation.

- | | |
|---|--|
| A. 0.3 cc. hypoxanthine : no vanillin. | E. 1.0 cc. vanillin : no hypoxanthine. |
| B. 0.3 cc. hypoxanthine + 0.2 cc. vanillin. | F. 0.5 cc. vanillin : no hypoxanthine. |
| C. 0.3 cc. hypoxanthine + 0.5 cc. vanillin. | G. 0.2 cc. vanillin : no hypoxanthine. |
| D. 0.3 cc. hypoxanthine + 1.0 cc. vanillin. | |

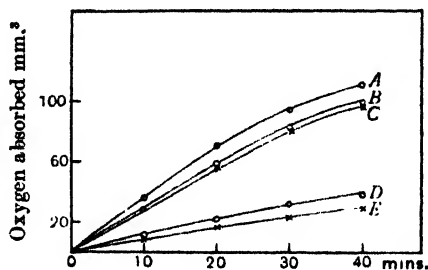


Fig. 14.

Fig. 14. Effect of piperonal on hypoxanthine oxidation.

- | | |
|--|----------------------------|
| A. 0.3 cc. hypoxanthine : no piperonal. | D. 0.5 cc. piperonal only. |
| B. 0.3 cc. hypoxanthine + 0.2 cc. piperonal | E. 0.2 cc. piperonal only. |
| C. 0.3 cc. hypoxanthine + 0.5 cc. piperonal. | |

In these experiments (Figs. 13 and 14) the concentration of vanillin was 0.5 % and of piperonal 0.25 %, both prepared in phosphate buffer of p_H 7.6. The Barcroft cups were made up as follows. The right-hand cup usually received a constant amount of 0.3 cc. hypoxanthine 0.2 % solution (unless otherwise stated) and varying amount of either vanillin or piperonal. 0.5 cc. of the 20 % caseinogen solution was kept in the small Keilin tubes and was tipped into both the cups after temperature equilibration, and this was taken as the zero time. The total volume was adjusted at the beginning to 3 cc. by the addition of phosphate buffer in both the cups. Control experiments were also made without any hypoxanthine.

SUMMARY.

1. Narcotics have no effect on xanthine oxidase or Schardinger enzyme (caseinogen preparation) in ordinary concentrations, either as regards oxygen absorption or anaerobic reduction of methylene blue.

2. Narcotics have a great inhibiting action on the succinic dehydrogenase system, the effect increasing with higher homologues of the same series. The degrees of inhibition of oxygen-uptake and methylene blue reduction agree with each other in the majority of cases. Nitriles have, however, no effect on methylene blue reduction, though they inhibit the oxygen-uptake strongly.

3. A competitive inhibition of hypoxanthine oxidation is observed in presence of vanillin and piperonal. This is considered to be a strong point in favour of the view that xanthine oxidase and Schardinger enzyme are identical.

In conclusion I desire to express my thanks to Dr D. Keilin and Dr Malcolm Dixon for their helpful advice and criticism and to Sir F. G. Hopkins for his kind interest in this work.

REFERENCES.

- Ahlgren (1926). *Skand. Arch. Physiol.* **47** (Supplement).
Batelli and Stern (1913, 1). *Biochem. Z.* **52**, 226.
—— — (1913, 2). *Biochem. Z.* **52**, 253.
Bernard (1885). *Leçons sur les phénomènes de la vie communs
aux animaux et aux végétaux.*
Coombs (1927). *Biochem. J.* **21**, 1259.
Dixon and Thurlow (1924, 1). *Biochem. J.* **18**, 971.
—— — (1924, 2). *Biochem. J.* **18**, 985.
Henderson (1930). *Physiol. Reviews*, **10**, 214.
Keilin (1929). *Proc. Roy. Soc. Lond. B* **104**, 206.
Loewe (1913). *Biochem. Z.* **57**, 161.
Vernon (1914). *Biochem. Z.* **60**, 202.
Warburg (1921). *Biochem. Z.* **119**, 134.
Wieland and Macrae (1930). *Liebig's Ann.* **483**, 217.

XCVII. OXIDATION-REDUCTION POTENTIALS OF HAEMOLYTIC STREPTOCOCCI.

II. EFFECT OF CATALASE.

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(Received April 30th, 1931.)

PEROXIDE formation has a marked effect on the electrode potential behaviour of bacterial cultures and it has been shown in previous communications that the course of development of reducing and oxidising conditions in cultures of the peroxide-forming organisms haemolytic streptococci [Hewitt, 1930, 1] and pneumococci [1930, 4] differs characteristically from that in cultures of the catalase-containing bacteria *C. diphtheriae* [1930, 2] and staphylococci [1930, 3]. Certain of the characteristics in the behaviour of pneumococcus disappear when peroxide accumulation is prevented by addition of catalase to the cultures [1931, 1]. Nevertheless differences still remain between the reducing activities of pneumococcus cultures containing catalase on the one hand and, say, staphylococcus cultures on the other.

Hydrogen peroxide is a common by-product in biological oxidation-reductions and has a very definite effect on electrode potentials. It is clear therefore that the presence or absence of catalase (or other peroxide-decomposing system) must have a pronounced effect on the potentials developed in bacterial cultures. It is important, however, to bear in mind that the presence or absence of other enzymes concerned in biological oxidation-reduction reactions must also have marked effects. McLeod [1930] has suggested that the phenomena of anaerobiosis may be ascribed to peroxide formation, but it is probable that other factors are involved [Hewitt, 1931, 2]. It is of interest, therefore, to determine the differences in the oxidation-reduction potentials developed in cultures of different organisms when peroxide formation is eliminated by the addition of catalase, and this short communication is an account of the effect of catalase on the oxidation-reduction potentials of cultures of haemolytic streptococci.

METHODS.

Electrode potentials of cultures were measured by means of the apparatus and methods previously described [1930, 1; 1931, 2]. The glossy variant of Aronson-Schnitzer strain of haemolytic streptococci [Todd, 1930, 1] was used and the inoculum of 0.1 cc. of a 24 hour broth culture was made into 8 cc. of medium in each experiment. Cultures were incubated at 37° and readings of the potential were made every 30 minutes during the first 12 hours' incubation.

The catalase preparation used was prepared from pig's liver by the method of Batelli and Stern [1904]. The liver was extracted with water, the catalase was precipitated by addition of alcohol, filtered off, redissolved in water, reprecipitated by alcohol, filtered off and dried *in vacuo*. Catalase broth was prepared by suspending the highly active catalase preparation in broth in the required concentration, and after standing for an hour the broth was filtered through a sterile Seitz filter. The catalase preparation did not dissolve completely, but the filtered broth decomposed hydrogen peroxide vigorously even after standing at room temperature for 2 months. 1 % and 0.2 % catalase broth were used, that is 1.0 or 0.2 g. of the catalase powder suspended in 100 cc. of broth.

RESULTS.

No differences were detected between the electrode potential of plain broth and that of broth to which the catalase preparation had been added 24 hours previously.

Stationary aerobic cultures (Fig. 1). In plain peptone infusion broth cultures

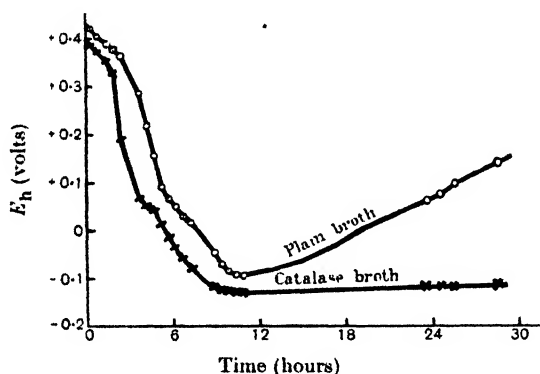


Fig. 1. Stationary aerobic cultures (peptone-infusion broth).

the potential fell to its minimum value of about -0.1 v. in 12 hours. The potential had risen to $+0.07$ v. in 24 hours and to $+0.16$ v. in 30 hours. This rapid rise in potential after the logarithmic phase of growth is characteristic of peroxide-forming bacteria. In broth containing 0.2 % of the liver catalase preparation the potential fell to a slightly lower level than that of the plain broth culture and after 30 hours' incubation was still below -0.11 v. The catalase had thus inhibited the usual rise in potential after the logarithmic phase of growth and had brought the behaviour of haemolytic streptococci more into line with that of staphylococci or *C. diphtheriae*. The behaviour of pneumococci in the presence of catalase was similar. The disappearance of reducing conditions after the logarithmic phase of growth is evidently associated with peroxide formation, although no peroxide can be detected in aerobic cultures under these conditions.

The presence of high concentrations of the catalase preparation in broth

(e.g. 1 %) produced a definite poisoning effect on the potentials, probably owing to the presence of extraneous substances such as haematin derivatives.

Aerated cultures (Fig. 2). Cultures were aerated in three-limbed cells by

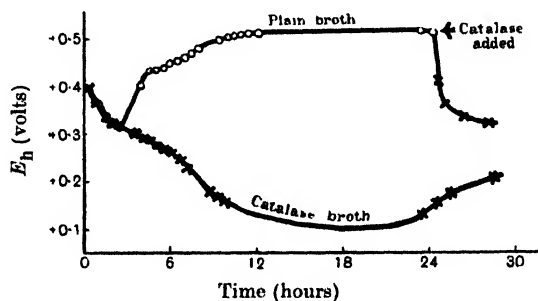


Fig. 2. Aerated digest-broth cultures.

the method previously described [1930, 1]. In the plain tryptic digest broth cultures the potential fell to a minimum level of +0.31 v. in 2.5 hours and then rose rapidly to +0.5 v., at which level peroxide may be detected in the culture. In the presence of 0.2 % catalase however the potential fell to a level of about +0.1 v. and after 30 hours' incubation had not risen above +0.2 v. The addition of catalase, therefore, had prevented peroxide formation, with the result that the potential fell to a much lower level and did not rise to the high level corresponding to peroxide formation. A similar effect was seen in the case of pneumococci [1931, 1].

As a matter of interest a small quantity of the catalase preparation was added to the aerated plain broth culture after 25 hours' incubation. There was evident effervescence of oxygen as the peroxide present was decomposed. Despite this vigorous oxygenation the potential fell rapidly. The fall was 0.1 v. within 10 minutes after the addition of the catalase, and the total fall in 4 hours was 0.2 v. Two conclusions may be drawn from this experiment; first, it is evident that peroxide formation accounted for the high potential since the addition of catalase resulted in an immediate fall; second, the relatively inert nature of molecular oxygen is demonstrated. Although hydrogen peroxide may be regarded as a reduction product of oxygen, yet peroxide has a much higher oxidising potential. The tendency to part with molecular oxygen evidently does not account for the oxidising effect of hydrogen peroxide.

DISCUSSION.

Bacterial peroxide formation is of considerable importance since it has been suggested as a criterion for the classification of bacteria, as an explanation of the behaviour of obligate anaerobes [McLeod, 1930] and as a factor in bacterial variation [Todd, 1930, 1, 2, 3]. The glossy variant of haemolytic streptococci is less sensitive to peroxide than the matt variants, hence there is a natural selection of glossy variants when peroxide is formed. When,

however, peroxide formation is inhibited by the presence of catalase the degradation of matt to glossy variants does not occur [Todd, 1930, 1]. The glossy variants are avirulent, whilst the matt form may be virulent or attenuated, and the virulence is maintained on long continued artificial cultivation only when the oxygen supply is abundant and peroxide accumulation in the culture is avoided [Todd, 1930, 2]. The effect of catalase on the oxidation-reduction conditions developed in cultures of haemolytic streptococci is, therefore, of more than academic interest.

Addition of catalase to the cultures inhibits peroxide formation by haemolytic streptococci and results in corresponding modifications of the electrode potential-time curves. In aerobic catalase broth cultures the potential remains at a low level long after the cessation of active proliferation and does not rise as in plain broth cultures. In aerated cultures the potential falls to a lower level in the presence of catalase and the usual high level corresponding to peroxide formation is not reached. The effect of catalase on cultures of haemolytic streptococci is therefore closely similar to that observed with pneumococci [1931, 1].

When a small quantity of the catalase preparation was added to an aerated culture of haemolytic streptococci in which peroxide had been formed the potential immediately fell very rapidly although oxygen was visibly liberated throughout the culture. This presents confirmatory evidence that

(1) the effect of the enzyme preparation on the potentials of growing cultures was due to catalase activity and not to extraneous factors;

(2) bacterial peroxide is closely similar to, if not identical with, hydrogen peroxide;

(3) peroxide has a much greater effect on the potential than molecular oxygen.

In many respects, cultures of haemolytic streptococci to which catalase has been added behave qualitatively like those organisms which produce their own catalase. Quantitative differences, however, still remain. Thus the potential-time curves of aerated cultures of pneumococci and haemolytic streptococci are entirely different from those of staphylococci and *C. diphtheriae*. When catalase is added to cultures of pneumococci and haemolytic streptococci the potential-time curves are now roughly similar in form to those of the catalase-containing bacteria; but the minimum values of potential reached vary widely, being approximately: pneumococci + 0.15 v., haemolytic streptococci + 0.05 v., staphylococci - 0.05 v. and *C. diphtheriae* - 0.15 v. These figures are only roughly comparable since they were obtained on different occasions with different samples of broth *etc.* They are sufficient, however, to indicate the great differences in reducing activity of these different bacteria. In aerobic cultures not subjected to special aeration the differences are much smaller, the minimum values being approximately pneumococci - 0.16 v., haemolytic streptococci - 0.17 v., staphylococci - 0.18 v. and *C. diphtheriae* - 0.20 v.

Thus, although the formation or non-formation of peroxide may be a valuable criterion for the classification of bacteria and may have important effects on bacterial variation, it should not be regarded as the only determining factor in bacterial behaviour.

SUMMARY.

1. Addition of catalase inhibits peroxide formation in cultures of haemolytic streptococci and the effect is closely similar to that observed with pneumococci.

2. Reducing conditions are maintained after the logarithmic phase of growth when catalase has been added to the culture.

3. The electrode potential falls to a lower level in aerated cultures when catalase is added, and the high level corresponding to peroxide formation is not reached.

4. Addition of catalase to a culture containing peroxide causes an immediate and rapid fall in potential despite the resulting vigorous oxygenation of the culture.

The author acknowledges his indebtedness to Dr R. G. White and Dr E. W. Todd for their continued help and encouragement.

REFERENCES.

- Batelli and Stern (1904). *Compt. Rend. Soc. Biol.* **57**, 374.
Hewitt (1930, 1). *Biochem. J.* **24**, 512.
—— (1930, 2). *Biochem. J.* **24**, 669.
—— (1930, 3). *Biochem. J.* **24**, 676.
—— (1930, 4). *Biochem. J.* **24**, 1551.
—— (1931, 1). *Biochem. J.* **25**, 169.
—— (1931, 2). Oxidation-reduction potentials in bacteriology and biochemistry, pp. 46-51. (London.)
McLeod (1930). A system of bacteriology, vol. 1, chap. vii. (Medical Research Council, London.)
Todd (1930, 1). *Brit. J. Exp. Path.* **11**, 368.
—— (1930, 2). *Brit. J. Exp. Path.* **11**, 469.
—— (1930, 3). *Brit. J. Exp. Path.* **11**, 480.

XCVIII. A METHOD FOR THE DETERMINATION OF SMALL QUANTITIES OF MIXED REDUCING SUGARS AND ITS APPLICATION TO THE ESTIMATION OF THE PRODUCTS OF HYDROLYSIS OF STARCH BY TAKA-DIASTASE.

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(Received May 1st, 1931.)

PRELIMINARY investigations on the carbohydrates in the developing apple have shown that the concentration of sugars in the fruit is less than 1 % during the first weeks of growth, and further, that the maximum starch concentration reached at any time is only about 2 %. Estimation of these carbohydrates by the usual methods would require the collection of a very large number of apples, especially in the early stages of development, when the fruit is small, with the resultant risk that the crop on any tree would be so seriously depleted that the development of the remaining fruit might be abnormal.

Since starch appears to be most satisfactorily estimated by determination of the glucose and maltose produced by hydrolysis with taka-diastrase, a method for the estimation of small quantities of reducing sugars has been investigated. With such a method, determinations of starch could be carried out during the development of the apple, and furthermore, a delicate method of estimation of reducing sugar would enable the size of the samples required for sugar determination on the young and very small fruits to be kept within reasonable limits.

Estimation of small amounts of reducing sugars.

In order to determine two mixed reducing sugars it is necessary to employ two methods of estimation, and to solve the pair of simultaneous equations obtained from the results of the determinations.

Hagedorn and Jensen [1923] have used the method of oxidation by alkaline ferricyanide for the determination of amounts of glucose ranging from 0.02 to 0.36 mg. in 2 cc. Hanes [1929] has applied this method to the estimation of quantities of glucose and maltose up to 3.8 mg. in 5 cc.; also, Macleod and Robison [1929] have shown that the oxidation of reducing sugars by hypiodite can be satisfactorily carried out in solutions of similar concentration.

The iodimetric method has already proved satisfactory for the determination of sugar in the apple if the oxidation is carried out on solutions containing about 0.06 g. sugar. It was therefore decided to investigate the oxidation of mixtures of glucose and fructose and of glucose and maltose by alkaline ferricyanide and alkaline iodine in concentrations of about 3 mg. per 5 cc.

Preparation of samples of pure sugar.

The glucose and fructose used were taken from the same samples as those employed by Archbold and Widdowson [1931] for an investigation of the iodimetric method.

Their rotatory powers and copper reducing values were determined:

Glucose.

$[\alpha]_D^{20}$ (10 % solution) observed 52.73°; calculated 52.74°.

Weight of glucose taken 10.000 g. per 100 cc.

Weight of glucose (Cu value) found 9.998 g. per 100 cc.

Ash < 0.01 %.

Fructose.

$[\alpha]_D^{27}$ (5.59 % solution) observed - 88.19°; calculated (using Vosburgh's [1920] formula) - 88.15°.

Weight of fructose taken 5.59 g. per 100 cc.

Weight of fructose (Cu value) found 5.52 g. per 100 cc.

Preparation of maltose. A sample of maltose, purchased as pure, was recrystallised twice by dissolving 25 g. in 200 cc. of 80 % alcohol heated in a water-bath under a reflux condenser. The solution was filtered, and sufficient alcohol was added to make 90 % concentration. The maltose was allowed to crystallise, filtered off and recrystallised in the same way. The maltose hydrate so obtained was dried *in vacuo* over sulphuric acid and then over phosphorus pentoxide until constant in weight, and finally dried at 50° until again constant in weight.

Copper reducing power (g. maltose hydrate per 100 cc. solution).

Calculated

Found

0.3771

0.3829

Optical rotation (calculated as anhydrous maltose).

$[\alpha]_D^{28}$ (4.7146 % solution)

Calculated 137.63 [Meissl, 1882]

Found 137.55

Oxidation of sugars by alkaline potassium ferricyanide.

The oxidation of the sugars by alkaline ferricyanide was carried out as described by Hanes, using sodium thiosulphate *N*/75 standardised against potassium iodate¹ solution and the following reagents:

¹ The potassium iodate was dried by heating at 100° for 1 hour. It was found that prolonged heating at 100° caused a slight discoloration of the salt. The thiosulphate was further standardised against potassium permanganate solution, and this gave a result differing only by 0.2 % from that given by the dried iodate.

A.	Potassium ferricyanide	8.25	} g. per litre
	Anhydrous sodium carbonate	10.6	
B.	Potassium iodide	12.5	} g. in 500 cc.
	Zinc sulphate	25.0	
	Sodium chloride	125.0	
C.	Acetic acid	7.5 %	

5 cc. sugar solution containing about 3 mg. sugar were measured into a glass specimen tube $1\frac{1}{4}" \times 4"$ and 5 cc. of solution A were added. The tube was covered with a glass lid and placed in a boiling water-bath for exactly 15 minutes. It was then cooled in water for 1 minute and in ice for 2 minutes, after which 5 cc. of solution B and 2 cc. of solution C were quickly added, and the iodine liberated was titrated with *N*/75 sodium thiosulphate solution. Two drops of a 1 % solution of soluble starch in saturated sodium chloride were used as indicator. The tubes were left immersed in ice till the contents were titrated in order to prevent any loss of iodine. It was found convenient to carry out the estimations in batches of six. Blank determinations were made by substituting 5 cc. of distilled water for the sugar solution, and all determinations were carried out in triplicate.

Hanes used boiling-tubes $1" \times 7"$ for his estimations, and apparently did not find it necessary to cool the tubes in ice to prevent loss of iodine. Using specimen tubes $1\frac{1}{4}" \times 4"$ however, a series of blank determinations has shown that a loss of iodine equivalent to 0.06 cc. *N*/75 thiosulphate occurred if the tube containing the liberated iodine was allowed to stand for 10 minutes at room temperature, while the loss was equivalent to 0.33 cc. *N*/75 thiosulphate at the end of an hour. If the estimations are carried out in batches of six, 10 to 15 minutes will elapse between the titration of the liberated iodine in the first tube and the sixth, so that a serious error may be introduced by loss of iodine unless the procedure for cooling described above is adopted. The results of the determinations are shown in Table I.

Table I. *Loss of iodine on standing at room temperature.*

			<i>N</i> /75 thiosulphate required cc.
Titrated immediately			9.02
Titrated after 10 min. at room temperature			8.96
"	30	"	8.79
"	60	"	8.69
"	10	in ice	9.02
"	60	"	8.99

Sobotka and Reiner [1930] have investigated the oxidation of various sugars by alkaline ferricyanide, and obtained the same results as Hanes for glucose and maltose, confirming his observation that the reducing power of glucose increases slightly as the concentration of sugar increases, while that of maltose is practically constant. They also investigated the oxidation of fruc-

tose, and obtained a constant value for the reducing power, but for mixtures of fructose and glucose they found a lower reducing power than that obtained for either sugar alone. Callow [1930] found for invert sugar a simple relation between the thiosulphate equivalent and the invert sugar reduced, and his result differs only slightly from that obtained by Sobotka and Reiner for mixtures of glucose and fructose. Similar determinations were repeated under the conditions described above on a series of glucose, maltose and fructose solutions, containing weights of sugar varying from 0.5 to 3.5 mg. in 5 cc.

The results are shown in Table II.

Table II. *Oxidation of glucose, maltose and fructose by alkaline ferricyanide.*

mg. glucose	N/100 thiosulphate \equiv ferricyanide reduced cc.	cc. N/100 thio- sulphate/mg. sugar
0.671	1.99	2.97
1.342	4.04	3.01
2.013	6.09	3.03
2.684	8.23	3.07
3.353	10.36	3.09
 mg. maltose (anhydrous)		
0.558	1.43	2.56
1.117	2.80	2.51
1.675	4.25	2.54
2.792	7.02	2.52
3.351	8.46	2.53
Mean value		2.53
 mg. fructose		
0.559	1.58	2.83
1.118	3.25	2.91
1.677	4.88	2.91
2.795	8.24	2.95
3.354	9.93	2.96

The results obtained show an increase in oxidation with concentration for glucose and fructose, and a constant value for maltose. For glucose and maltose the results are slightly higher than those found by Hanes, but repeated determinations, using every precaution to prevent loss of iodine always gave the same result. For fructose a definitely lower series of values is obtained than that observed by Sobotka and Reiner. In order to confirm this result determinations were carried out on invert sugar, obtained by dissolving 0.95 g. of sucrose in 150 cc. of water, boiling with 30 cc. of 0.5 *N* HCl for 1 minute, cooling, neutralising and diluting as required. The values obtained for the reducing power of the invert sugar estimated by oxidation with alkaline ferricyanide agreed very closely with those for mixtures of equal quantities of fructose and glucose and with the "mean value" for the sugars alone. The results are shown in Table III.

In describing the preparation of his sample of glucose, Hanes states that the sugar was dried *in vacuo* over concentrated H_2SO_4 for 48 hours. It has been found, however, that after 4 months *in vacuo* over P_2O_5 , glucose still contains

2-3 % of water which can be driven off at 100°. Further, Hanes gives a value for the specific rotation of his sample of glucose $[\alpha]_D^{20} = +52.2^\circ$ in 10 % solution.

Table III. *Oxidation of mixtures of equal quantities of glucose and fructose by alkaline ferricyanide.*

(N/100 thiosulphate \equiv 1 mg. sugar.)			
mg. sugar	Mean value for glucose and fructose estimated separately cc.	Mixture of glucose and fructose cc.	Invert sugar cc.
0.5	2.91	—	2.87
1.0	2.95	2.93	2.94
2.0	—	2.96	2.97
3.0	3.02	3.05	3.02

According to Tollens [1884] the theoretical value for a 10 % solution is 52.75°. (In the theoretical value 52.5° given by Hanes there is no correction for the concentration of the solution.) Both these observations suggest that his sample of glucose was not completely dry. Assuming that the glucose still contained 2 % of water his results would agree very closely with those shown in Table II.

Sobotka and Reiner give no details of the preparation of their samples of sugar, and it is difficult to account for the high reducing power obtained by them for fructose.

Oxidation of mixtures of glucose and maltose and of glucose and fructose by alkaline ferricyanide.

A series of determinations was next carried out on solutions containing mixtures of glucose and maltose and of glucose and fructose, the relative proportions of the sugars and the total concentration of the sugar in the solution being varied. For glucose and maltose the total sugar content of the solution was varied from 1 to 3 mg. in 5 cc., while the ratio of glucose to maltose was varied from 3:1 to 1:3. Then from pairs of results, the ferricyanide factors for glucose and maltose in the presence of one another were calculated by the method shown below.

	Wt. maltose (anhydrous) in 5 cc. mg.	Wt. glucose in 5 cc. mg.	Total wt. sugar in 5 cc. mg.	N/100 thiosulphate \equiv ferricyanide used by 5 cc. cc.
1	1.117	1.071	2.188	5.95
2	0.558	1.607	2.165	6.21
	1.117M + 1.071G = 5.95		0.653M + 0.598G = 3.32	
	0.558M + 1.607G = 6.21		0.653M + 1.795G = 6.94	
			<hr/> 1.197G = 3.62	
			G = 3.02	
	1.607G = 4.85			
	0.558M = 1.36			
	M = 2.44			
G.	Glucose factor = 3.02		where M and G stand for the maltose and glucose factors respectively, i.e. cc. N/100 thiosulphate/mg. sugar.	
M.	Maltose factor = 2.44			

The values for the series of estimations are shown in Table IV. The calculation shown above is made for experiment 5. It will be seen from the mean values obtained that the oxidation of one sugar is not affected by the presence of the other.

Table IV. *Oxidation of mixtures of glucose and maltose by alkaline ferricyanide.*

(N/100 thiosulphate \equiv 1 mg. sugar.)		
	Glucose cc.	Maltose cc.
1.	3.09	2.47
2.	2.93	2.52
3.	3.05	2.55
4.	2.96	2.59
5.	3.02	2.44
	<hr/>	<hr/>
Mean	3.01	Mean 2.53

A similar set of determinations was carried out on mixtures of glucose and fructose, the ratio of glucose to fructose being varied from 1:1 to 1:5, whilst the total concentration of sugar in the solution used for estimation was maintained at about 3.0 mg. The factors for the oxidation of glucose and fructose in the presence of one another by alkaline ferricyanide were calculated from pairs of results as for the glucose and maltose, and a series of the results obtained is shown in Table V.

Table V. *Oxidation of mixtures of glucose and fructose by alkaline ferricyanide.*

(N/100 thiosulphate \equiv 1 mg. sugar.)		
	Glucose cc.	Fructose cc.
	3.11	2.99
	3.13	2.97
	3.15	2.97
	<hr/>	<hr/>
Mean	3.13	Mean 2.97

The factors for both sugars are seen to be slightly higher than those obtained for either sugar alone, so that there appears to be a slightly increased oxidation of these sugars in the presence of one another if the ratio of fructose to glucose is greater than 1. It was found however that the use of the factors obtained for the sugars in their mixtures, when combined with the results obtained for the oxidation by hypiodite, gave a value for the amount of fructose in a given solution which only differed by 1 % from the value for fructose when the factors obtained for the sugars alone were substituted in the calculation, while the calculated amount of glucose remained unaltered.

Oxidation of glucose, maltose and fructose by hypiodite at 1°.

For the oxidation of glucose, maltose and fructose by hypiodite the following reagents are required:

Sodium thiosulphate	N/75
Iodine in potassium iodide	N/40
Sodium hydroxide	0.3 %
Sulphuric acid	N/4

Samples of 5 cc. of sugar solution, containing about 3 mg. sugar are measured out into 2 oz. stoppered bottles which are left at 1° until the solutions have cooled to this temperature. 5 cc. N/40 iodine solution are then added followed by 2 cc. 0.3 % sodium hydroxide, and the bottles are placed in a water-bath maintained at 1° until the oxidation is complete. The stock solutions of iodine and sodium hydroxide are always kept at 1°, so that the temperature of the reaction mixture is not raised by their addition. When the reaction is complete the bottles are removed from the bath, 2 cc. N/4 sulphuric acid are added to each and the excess of iodine is titrated with N/75 sodium thiosulphate solution. All estimations are carried out in duplicate and blank determinations are made by substituting 5 cc. water for the sugar solution.

The oxidation of glucose to gluconic acid by hypiodite at 1° has been found to be complete in 2 hours for a concentration of glucose about 0.06 % [Archbold and Widdowson, 1931]. The time of reaction for small quantities of glucose and maltose under the conditions described above was determined by allowing 5 cc. of solutions containing about 3 mg. of the sugars to react with hypiodite at 1° for varying lengths of time. It was found that the oxidation of both sugars is complete in 2 hours and this period was accordingly used for all subsequent estimations.

The results are shown in Table VI.

Table VI. *Oxidation of glucose and maltose by hypiodite.*
Time of reaction at 1°.

Time of oxidation hours	N/100 thiosulphate ≡ I used cc.	cc. N/100 thio- sulphate/mg. sugar
Glucose 3.353 mg. in 5 cc.		
0½	3.35	1.00
1	3.61	1.08
1½	3.71	1.11
2	3.72	1.11
	Theoretical value	1.11
Maltose 3.776 mg. (anhydrous) in 5 cc.		
0½	2.08	0.55
1	2.15	0.57
1½	2.21	0.58
2	2.23	0.59
	Theoretical value	0.59

Estimations were also carried out on solutions containing mixtures of glucose and maltose in varying proportions, and from pairs of results the iodine factors of the two sugars in the presence of one another were calculated by a method similar to that used for the calculation of the ferricyanide factors. A summary of the results is shown in Table VII. From the mean results it will be seen that the oxidation of one sugar by hypoiodite is not affected by the presence of the other.

Table VII. *Oxidation of mixtures of glucose and maltose by hypoiodite.*

(N/100 thiosulphate \equiv 1 mg. sugar.)			
Glucose		Maltose	
cc.		cc.	
1.11		0.59	
1.09		0.60	
1.11		0.62	
1.13		0.56	
1.13		0.56	
Mean		1.11	Mean 0.59

The oxidation of small quantities of fructose alone by iodine and sodium hydroxide at 1° for 2 hours showed that the mean value of N/100 thiosulphate equivalent to 1 mg. sugar was 0.02 cc. For mixtures of glucose and fructose the oxidation of fructose was too small to be detected when less than 3 mg. of this sugar was present. For quantities of fructose above 4.0 mg. the fructose factor decreased with increasing quantities of fructose. The results are shown in Table VIII.

Table VIII. *Oxidation of fructose by hypoiodite at 1° for 2 hours.*

	Fructose mg.	N/100 thiosulphate equivalent to fructose cc.	N/100 thiosulphate \equiv 1 mg. fructose cc.			
Oxidation of fructose alone.						
	0.559	0.01	0.018			
	1.118	0.03	0.027			
	1.677	0.04	0.024			
	2.795	0.05	0.018			
	3.354	0.06	0.018			
		Mean value	0.021			
	Fructose mg.	Glucose mg.	N/100 thiosulphate \equiv glucose (calculated) cc.	N/100 thiosulphate \equiv glucose + fructose (found) cc.	N/100 thiosulphate \equiv fructose cc.	N/100 thiosulphate \equiv 1 mg. fructose cc.
Oxidation of fructose in the presence of glucose.						
	1.677	1.607	1.78	1.78	—	—
	2.236	1.071	1.19	1.19	—	—
	2.795	0.536	0.59	0.60	—	—
	0.559	0.536	0.59	0.60	—	—
	1.118	1.071	1.19	1.18	—	—
	0.839	0.161	0.18	0.17	—	—
	1.677	0.321	0.36	0.35	—	—
	4.974	1.281	1.42	1.56	0.14	0.028
	7.461	1.921	2.13	2.29	0.16	0.021
	14.922	3.842	4.26	4.46	0.20	0.013

The oxidation of fructose by hypoiodite can therefore be neglected in calculating the quantities of the sugars in solutions containing mixtures of glucose and fructose in which the amount of fructose is less than 3 mg.; the iodine value can therefore be taken as a measure of the glucose present.

Macleod and Robison [1929] applied the iodimetric method to small quantities of reducing sugars and obtained a considerable oxidation of fructose (9 % in 20 min. at 21°) when sodium hydroxide was used as alkali. If sodium carbonate were used instead of the sodium hydroxide, however, fructose was oxidised only to a very small extent. The small oxidation of fructose (less than 2 %) here obtained when sodium hydroxide was used as alkali, as compared with the higher oxidation found by Macleod and Robison is probably due to the much lower temperature employed. Since satisfactory results could be obtained with sodium hydroxide at 1° its replacement by sodium carbonate was thought to be unnecessary.

The fructose-glucose ratio in the apple rarely exceeds 4:1, and in the young fruit for which this method was to be used the fructose and glucose are in approximately equal proportions, so a dilution of the cleared apple extract to bring it to a concentration of 3 mg. sugar per 5 cc. necessarily reduces the quantity of fructose present to a level at which its presence may be neglected.

The oxidation of solutions containing known weights of glucose and fructose was then carried out by both alkaline ferricyanide and hypoiodite, and by means of simultaneous equations the weights of glucose and fructose were calculated

$$1.11x = \text{cc. } N/100 \text{ thiosulphate} \equiv \text{iodine reduced by 5 cc. sugar solution}$$

$$3.13x + 2.97y = \text{cc. } N/100 \text{ thiosulphate} \equiv \text{ferricyanide reduced by 5 cc. sugar solution}$$

where x and y are the weights of glucose and fructose in 5 cc. of solution.

The results given in Table IX show a maximum error of 2 % on the weight of fructose present, while the recovery of glucose is quantitative.

Table IX. *Oxidation of mixtures of glucose and fructose by alkaline ferricyanide and hypoiodite.*

	Glucose taken mg.	Glucose found mg.	Fructose taken mg.	Fructose found mg.
1	1.458	1.459	1.809	1.845
2	0.972	0.973	1.809	1.822
3	0.486	0.486	1.809	1.801
4	0.486	0.486	3.015	3.050

In sugar solutions containing sucrose estimations of the total sugar present in the solutions can be carried out by hydrolysing the sucrose with hydrochloric acid and re-estimating the reducing sugars by both methods of oxidation. Citric acid must not be used for the inversion since it is oxidised both by hypoiodite and by alkaline ferricyanide.

The combination of the two methods of oxidation, by alkaline ferricyanide and by hypoiodite, thus appears to give satisfactory results for the estimation of small quantities of glucose and fructose and glucose and maltose, and could probably be applied to mixtures of any two sugars provided that the difference

between the ratio of the factors of the two sugars obtained from each method of estimation is sufficiently large. The method is convenient since the same thiosulphate solution is used for both sets of titrations.

Hinton and Macara [1924] have shown that unreliable results are obtained for iodimetric determinations of quantities of glucose of about 0.06 g. unless there is added more than twice the quantity of iodine required to oxidise the sugar. Macleod and Robison [1929] state that they obtain satisfactory results for about 1 mg. sugar if 3-4 times the theoretical quantity of iodine is present.

Since the ferricyanide factor for glucose is about 3 times the iodine factor for quantities of sugar of about 3 mg., and for maltose the ferricyanide factor is 5 times the iodine factor, if aliquot samples of the same solution are used for oxidation by both methods under the conditions described, the quantity of iodine present is always more than three times the theoretical quantity required to oxidise the sugar present, so this condition is automatically fulfilled.

*Investigation of the use of taka-diastrase for the estimation
of small quantities of starch.*

The hydrolysis of starch in plant materials by taka-diastrase instead of by mineral acid was investigated by Davis and Daish [1914], and it is now generally recognised that the older method of starch hydrolysis by boiling with dilute hydrochloric acid is quite valueless, because plant material contains a considerable quantity of pentose polymerides and other substances which yield reducing sugars when boiled with dilute acid. In addition, Davis and Daish pointed out that a destruction of glucose occurs on prolonged boiling with acid. This would introduce an error even if the preliminary hydrolysis were carried out with malt diastrase since a subsequent acid hydrolysis is necessary to convert the dextrans which are produced to reducing sugars.

Davis and Daish showed that under suitable conditions, starch is converted quantitatively into glucose and maltose by taka-diastrase, and that therefore no subsequent acid hydrolysis is necessary. The glucose and maltose present in the hydrolysis mixture were estimated by a copper reduction method and polarimetrically, and by means of simultaneous equations the quantities of glucose and maltose present were calculated, and hence the amount of starch. They found that the optimum conditions for the hydrolysis were 24 hours at 38°.

Horton [1921] repeated the work of Davis and Daish both on pure starch and on starch in the grain of wheat. His first result on pure potato starch was satisfactory, but he was apparently unable to repeat this, and the results he obtained in all later experiments gave a recovery of starch which was too low. He suggests that the conversion of starch to glucose and maltose is not quantitative but that a small quantity of dextrin still persists. He also finds a large variation in the glucose-maltose ratio, and concludes that different preparations of the enzyme vary in their maltase content.

Tottingham and Gerhardt [1924] obtained a smaller recovery of starch

with taka-diastrase than with ptyalin from woody tissues and their difficulties appear to be similar to those encountered by Horton.

Thomas [1924] has stated that the ratio of glucose to maltose obtained by hydrolysis of starch with taka-diastrase is constant, and therefore an estimation of the sugars in the hydrolysate by the picric acid reduction method gives a measure of the starch present.

Bish [1929] used taka-diastrase in the determination of small quantities of starch in bracken rhizome, and he estimated the resulting mixture of sugars by Shaffer and Hartmann's [1921] copper reduction method. He claims that the results so obtained give comparative values for the starch content of the tissue. This method is only reliable if there is no variation in the glucose-maltose ratio.

In view of the differences of opinion with regard to the hydrolysis of starch by taka-diastrase, the hydrolysis of pure starch by this enzyme has been investigated, and the application of the methods of oxidation by alkaline ferricyanide and by hypiodite to the products of hydrolysis has been studied.

Purification of starch. A sample of maize starch was washed with cold water, alcohol and ether, and was dried at 100°. Duplicate weighed samples were then dried at 120°, one *in vacuo* and the other under atmospheric pressure, for 16 hours. It was found that more than 99.9 % of the water remaining after drying at 100° was removed at 120° at atmospheric pressure, and that after drying at 100° to constant weight the starch contained 3.575 % of water. Quantities of 0.3, 0.2 and 0.1 g. of purified starch, dried at 100°, were weighed out into 200 cc. conical flasks, and 100 cc. distilled water were added to each. The flasks were then heated in a boiling water-bath for half an hour, cooled to room temperature and 10 cc. of freshly prepared 1 % taka-diastrase solution, 0.05 cc. of 5 % acetic acid and a little toluene were added to each. It had previously been found that 5 cc. of a 0.3 % solution of taka-diastrase was sufficient for the complete hydrolysis of 0.3 g. of purified starch, but a preliminary trial showed that the minimum quantity of the enzyme required for the hydrolysis of a corresponding weight of starch in apple tissue was 0.1 g., so this quantity was adopted for the investigation of the hydrolysis of the purified starch.

The flasks were plugged with cotton wool and placed in an incubator at 38° for 24 hours. After removal from the incubator the solutions were heated to boiling to destroy the enzyme and to remove the toluene. They were then cooled and diluted to 500 cc. and the sugars in 5 cc. of the solution estimated as already described. A blank determination was carried out omitting the starch. The thiosulphate titration for the hydrolysed starch solution was corrected for the titration for the blank determination for the taka-diastrase before calculation of results. Then by means of simultaneous equations the glucose and maltose present were calculated

$$1.11x + 0.59y = \text{cc. } N/100 \text{ thiosulphate} \equiv \text{iodine reduced by 5 cc. hydrolysate}$$

$$3.01x + 2.53y = \text{cc. } N/100 \text{ thiosulphate} \equiv \text{ferricyanide reduced by 5 cc. hydrolysate}$$

where x and y are mg. of glucose and maltose present in 5 cc. of the hydrolysis mixture. The amount of starch originally present was calculated by multiplying the estimated glucose by 0.9 and the maltose by 0.9479, and adding the products. An example of the calculation of results is shown below.

Calculation of results.

Wt. of starch dried at 100° = 0.1880 g.

Wt. of dry starch = 0.1813 g.

Diluted hydrolysate to 500 cc. Estimated sugar in 5 cc.

Thiosulphate (0.01303 N) \equiv ferricyanide reduced by 5 cc. of solution = 5.94 cc.

Thiosulphate (0.01303 N) \equiv ferricyanide reduced by 5 cc. of blank determination on taka-dia-stase = 1.51 cc.

Difference 4.43 cc.

Thiosulphate (0.01303 N) \equiv iodine reduced by 5 cc. of solution = 2.05 cc.

Thiosulphate (0.01303 N) \equiv 5 cc. of blank determination on taka-dia-stase = 0.52 cc.

Difference 1.53 cc.

$N/100$ thiosulphate \equiv ferricyanide reduced by hydrolysed starch in 5 cc. solution = 5.77 cc.

$N/100$ thiosulphate \equiv iodine reduced by hydrolysed starch in 5 cc. solution = 1.99 cc.

Then if x and y are the mg. glucose and maltose present in 5 cc. solution

$$\begin{array}{rcl} 1.11x + 0.59y & = & 1.99 \\ 3.01x + 2.53y & = & 5.77 \end{array}$$

$$3.341x + 1.776y = 5.990$$

$$3.341x + 2.808y = 6.405$$

$$1.032y = 0.415$$

$$y = 0.402$$

$$0.59y = 0.24$$

$$1.11x = 1.75$$

$$x = 1.577$$

$$\begin{aligned} \text{Starch originally present in 500 cc. solution} &= \frac{(1.577 \times 0.9) + (0.402 \times 0.9479)}{10} \text{ g.} \\ &= 0.1800 \text{ g.} \end{aligned}$$

It was found that under these conditions more than 99 % of the starch could be accounted for; a series of results is shown in Table X.

Table X. *Hydrolysis of purified maize starch with taka-dia-stase.*

	Starch taken g.	Starch found g.	Starch found %	Glucose/ maltose
1	0.3184	0.3158	99.18	4.69
2	0.1813	0.1800	99.28	3.92
3	0.1431	0.1426	99.65	2.74
4	0.1386	0.1376	99.28	4.96

The glucose/maltose ratio obtained was higher than that found by Davis and Daish and was not constant even with two samples of the same preparation of enzyme. This bears out the observations made by Horton in this regard, though the variation obtained is not so great as that recorded by him. No indication of the persistence of dextrin in the hydrolysate as suggested by Horton was obtained, and it appears that the only products of hydrolysis are glucose and maltose.

The effect of clearing glucose-maltose and hydrolysed starch solutions with basic lead acetate and sodium phosphate.

Since this method for the estimation of starch was intended to be applied to apple tissue, a preliminary investigation was carried out on the effect of clearing the solution containing hydrolysed starch derived from this material. This work showed that satisfactory results could not be obtained unless the solutions were cleared before estimation of the sugars, so the effect of clearing on glucose-maltose solutions was investigated.

A solution containing about 0.3 g. glucose and 0.3 g. maltose was diluted to 1000 cc. and three aliquots of 200 cc. were further diluted to 250 cc. To one was added 0.5 cc. of basic lead acetate solution, followed by 1.5 cc. of saturated sodium phosphate, and to another was added 1.0 cc. of basic lead acetate and 3.0 cc. sodium phosphate before diluting to 250 cc. The third was diluted to 250 cc. without clearing. The solutions were filtered, and the sugar in 5 cc. of each was estimated by oxidation with alkaline ferricyanide. It was found that no loss of sugar occurred when clearing was carried out with these reagents. This agrees with results obtained with glucose-fructose solutions on the macro-scale [Archbold and Widdowson, 1931]. The results are shown in Table XI.

Table XI. *Effect of clearing glucose-maltose solutions with basic lead acetate and sodium phosphate.*

	N/75 thiosulphate \equiv ferricyanide reduced cc.
Uncleared	6.25
Cleared 0.5 cc. basic lead acetate	6.24
Cleared 1.0 cc. basic lead acetate	6.26

The effect of clearing on solutions obtained by hydrolysis of starch with taka-diastase was next investigated. 0.3 g. starch was hydrolysed as already described, and the solution diluted to 500 cc.; 200 cc. were cleared with basic lead acetate and sodium phosphate, filtered, and diluted to 250 cc. A second 200 cc. were diluted to 250 cc. without clearing. A similar pair of experiments was carried out on taka-diastase and 100 cc. of water, with no starch added. The reducing power of the sugar in 5 cc. of all four solutions was estimated by oxidation with alkaline ferricyanide and hypiodite. The results are shown in Table XII.

Table XII. *Effect of clearing solutions of starch hydrolysed by taka-diastase.*

Results expressed as N/75 thiosulphate \equiv ferricyanide or iodine reduced by 5 cc. solution.

	Ferricyanide		Iodine	
	Cleared cc.	Uncleared cc.	Cleared cc.	Uncleared cc.
Taka-diastase blank	0.58	0.60	0.22	0.22
Starch	4.44	4.45	1.52	1.52

It was found that there was no precipitate when the lead acetate was added in either case although the solution became slightly turbid, and also, there was little or no difference between the values for the cleared and uncleared solutions. Hence it is unnecessary to clear solutions obtained by hydrolysing samples of pure starch before estimating the sugars. The presence of oxidisable material other than sugar in the aqueous extract from alcohol-insoluble apple residue after enzyme hydrolysis, however, necessitates clearing, but no loss of sugar occurs during the process.

It is thus evident that satisfactory results for starch can be obtained provided that no other polysaccharide present in the tissue under consideration is attacked by the enzyme. This point will be dealt with in a later paper.

Effect of boiling coloured apple extracts with "Suchar" on the ferricyanide and iodine oxidations.

It has previously been stated [Archbold and Widdowson, 1931] that the solutions from the evaporated alcoholic extracts from apples, after clearing with basic lead acetate and sodium phosphate, are colourless when first prepared, but gradually develop a brown colour on standing. They have also been shown to contain some material, other than sugar, which is oxidised by hypiodite, but which has no effect on the copper reduction value of the solution. This oxidisable material can be removed by boiling the solution with charcoal before the iodine estimation is carried out. Solutions prepared from the alcoholic extracts of young apples are coloured immediately after clearing and become considerably darker on standing than those prepared from the more mature fruit. Accordingly, before proceeding to a series of estimations of the fructose and glucose in the extracts from the young apples, the effect on the ferricyanide and iodine oxidations of decolorising the brown solutions by boiling with charcoal was investigated. Coloured solutions were boiled for different numbers of times with "Suchar," a preparation of charcoal, and the sugars in the coloured and colourless solutions were estimated by both methods of oxidation. A difference of 0.66 cc. was obtained between the thiosulphate titrations for the ferricyanide oxidation of the coloured and the decolorised solutions, while the difference in the titrations for the iodine estimation was 0.24 cc. Decoloration is therefore necessary for both estimations. From the results obtained the apparent glucose and fructose present were calculated, and the results for one solution are shown in Table XIII.

Table XIII. *Effect of boiling with "Suchar" on a coloured apple extract.*

	% in apple	
	Glucose	Fructose
Unboiled	2.81	6.19
Boiled three times with "Suchar" (colourless)	2.37	6.22
" five " "	2.37	6.20

The effect of boiling with "Suchar" on solutions of fructose and glucose was next investigated to see if a measurable loss of sugar occurred during the process. Solutions of glucose and of glucose-fructose in the ratio 1:4, were left unboiled, boiled once, and boiled a number of times with "Suchar" and estimated by both ferricyanide and iodine methods.

The thiosulphate equivalent to the iodine reduced by 5 cc. of the solution decreased from 3.45 cc. for the unboiled solution to 3.35 cc. for the solution after boiling six times with "Suchar." The ferricyanide value also decreased from 9.71 cc. to 9.42 cc. This decrease in titration was equivalent to a 3 % loss of sugar. The results are shown in Table XIV.

Table XIV. *Effect of boiling with "Suchar."*

		mg. in 5 cc. solution	
Glucose		Glucose	Fructose
	Unboiled	3.102	—
	Boiled once	3.064	—
	Boiled six times	3.009	—
Glucose + fructose			
	Unboiled	0.838	3.071
	Boiled once	0.838	3.030
	Boiled four times	0.838	3.003

In the case of the glucose-fructose solution, in which the glucose only comprises about $\frac{1}{5}$ of the total weight of sugar present, there is no apparent loss of glucose after boiling the solution four times with "Suchar," but a loss of fructose of about 5 % occurs. Hence after boiling the solution four times with "Suchar" the ferricyanide value shows a decrease of 0.20 cc. while there is no change in the iodine value, since the oxidation of fructose by hypiodite is negligible. In this case the effect of the adsorption of glucose by the "Suchar" is too small to be detected in the iodine titration, and the loss of reducing sugar appears to be entirely due to adsorption of fructose. This result is similar to that found for more concentrated sugar solutions, when the iodine value decreases 0.8 % after six boilings with "Suchar", while the reducing power estimated by copper reduction shows a loss of 5 % [Archbold and Widdowson, 1931].

The decrease in both the iodine and the ferricyanide values found after boiling the coloured apple extract with "Suchar" is much larger than can be accounted for by adsorption of sugar by the charcoal. From these results it was concluded that coloured solutions must be decolorised by boiling with charcoal before the sugars in them are estimated by alkaline ferricyanide or hypiodite.

Determinations were carried out on a number of cleared solutions from evaporated alcoholic extracts of apples after boiling with "Suchar" till colourless, and diluting suitably for estimation. The values obtained for the percentage of glucose and fructose in the apple, determined by oxidation with alkaline ferricyanide and hypiodite, as compared with the results for the same

samples estimated on a larger scale by copper reducing power and iodimetrically are given in Table XV.

Table XV. *Determination of glucose and fructose in apple extracts.*

	% sugar in apple tissue (wet weight).					
	Estimated by copper reduction and oxidation by hypoiodite (sugar solution 0.2 %)			Estimated by oxidation with alkaline ferricyanide and hypoiodite (3 mg. sugar per estimation)		
	Glucose	Fructose	Reducing sugar	Glucose	Fructose	Reducing sugar
1	1.28	2.70	3.98	1.23	2.77	4.00
2	1.19	3.32	4.51	1.17	3.32	4.49
3	1.28	3.77	5.05	1.22	3.74	4.96
4	1.26	3.98	5.24	1.21	4.08	5.29
5	1.46	4.25	5.71	1.39	4.30	5.69
6	1.58	4.36	5.94	1.51	4.38	5.80

Although with pure sugar solutions a loss of fructose and glucose occurs on boiling the solutions with charcoal, it will be observed that the results obtained for the total reducing sugar determined by copper reduction on the coloured unboiled apple extracts agree with those obtained on the decolorised solution by oxidation with ferricyanide, suggesting that the coloured substance is preferentially adsorbed by the charcoal. It does not therefore appear necessary to make a correction for any loss of sugar which occurs during decoloration. The percentage of glucose calculated from the small scale estimations is always slightly lower than that obtained from the more concentrated solutions. The differences are in no case more than 5 %.

SUMMARY.

The Hanes modification of the Hagedorn and Jensen method for the determination of reducing sugars by oxidation with alkaline ferricyanide, when combined with the iodimetric method, has been satisfactorily applied to the estimation of small quantities of mixtures of glucose and fructose and of glucose and maltose.

These methods have also been applied to the determination of the glucose and maltose obtained by the hydrolysis of starch by taka-diastrase. Tests with pure starch showed that the method was accurate to within 1 %.

It has been shown that there is no loss of sugar when glucose-maltose solutions are cleared with basic lead acetate and sodium phosphate.

It has been shown that cleared, coloured solutions obtained from alcoholic extracts of young apples should be boiled with a preparation of charcoal such as "Suchar" before estimating the fructose and glucose by oxidation with alkaline ferricyanide and hypoiodite.

The author wishes to take this opportunity of thanking Miss H. K. Archbold for her valuable suggestions and advice.

REFERENCES.

- Archbold and Widdowson (1931). *Biochem. J.* **25**, 101.
Bish (1929). *Biochem. J.* **23**, 31.
Callow (1930). *Biochem. J.* **24**, 57.
Davis and Daish (1914). *J. Agric. Sci.* **6**, 152.
Hagedorn and Jensen (1923). *Biochem. Z.* **135**, 46.
Hanes (1929). *Biochem. J.* **23**, 99.
Hinton and Macara (1924). *Analyst*, **49**, 2.
Horton (1921). *J. Agric. Sci.* **11**, 240.
Macleod and Robison (1929). *Biochem. J.* **23**, 517.
Meissl (1882). *J. prakt. Chem.* (2) **25**, 114.
Shaffer and Hartmann (1921). *J. Biol. Chem.* **45**, 349.
Sobotka and Reiner (1930). *Biochem. J.* **24**, 394.
Thomas (1924). *J. Amer. Chem. Soc.* **46**, 1670.
Tollens (1884). *Ber. deutsch. chem. Ges.* **17**, 2234.
Tottingham and Gerhardt (1924). *J. Ind. Eng. Chem.* **16**, 139.
Vosburgh (1920). *J. Amer. Chem. Soc.* **43**, 219.

XCIX. THE RESPIRATION OF *B. COLI COMMUNIS*.

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(Received May 1st, 1931.)

COOK, HALDANE AND MAPSON [1931] investigated the oxygen-uptake of toluene-treated *B. coli* in presence of succinate, lactate, and formate. The toluene treatment restricts the oxidation of the first two substrates to a single stage, and thus renders the interpretation of the results easy. But it might be objected that the bacteria had been damaged by the toluene. For example the conclusion was drawn that the oxygenases concerned in the oxidation of the above substrates differed in their sensitivities to CO and HCN. This might conceivably have been due to the fact that, for reasons depending on cell structure, some were more accessible than others to toluene, and therefore more altered by it. We have therefore investigated the oxidation of several substances by the intact organism, besides making a few more observations in presence of toluene. The technique used was the same as that of Cook, Haldane and Mapson. Our N.T.C. strain of bacteria was used in *M*/15 phosphate buffer¹ at p_H 6.3 for formate oxidation, 7.6 for other substrates. All substrates were present in *M*/60 concentration, which is sufficient to give a maximum rate of oxidation in each case. Barcroft manometers were used throughout, and all experiments were carried out at 16°. When substrates were added, a bacterial suspension without substrate was placed in the left-hand cup. All experiments were done in duplicate.

KINETICS OF OXYGEN-UPTAKE.

In the absence of added substrate the rate of oxygen-uptake usually fell off during the first hour, and then became constant. (Fig. 1.)

In the presence of formate the curves obtained were very similar to those found in presence of toluene (Fig. 2). After 30 minutes the rate of oxygen-uptake became constant. If the experiments were continued long enough it fell off, but never increased. But in presence of glucose, succinate, and lactate, the curves obtained were concave upwards, *i.e.* the rate of oxygen-uptake increased with time, and ultimately became steady (Figs. 3, 4, 5). The reason for this is quite clear. The lactate, to take one example, is oxidised to pyruvate. This is not further oxidised in presence of toluene, so that the rate of oxygen

¹ The concentration of phosphate was incorrectly given in our former paper. 1.0 cc. of 0.2 *M* buffer solution was present in 3.0 cc. of mixture.

consumption does not rise. But the normal organism oxidises it. However, the amount of pyruvate produced in the first half hour or so at 16° is not nearly sufficient to saturate the catalysts concerned in its oxidation, so it, and prob-

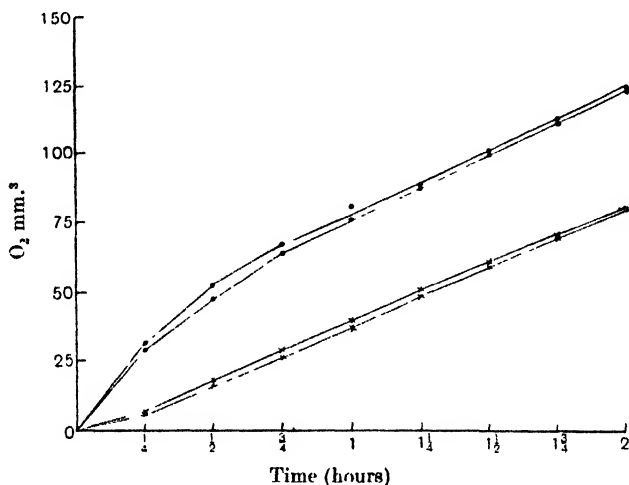


Fig. 1. O_2 consumption of washed *B. coli* (2.86 mg. N) in presence of air (upper curves) and a mixture containing 9 volumes of CO to 1 volume of O_2 (lower curves). p_H 7.6, 16° .

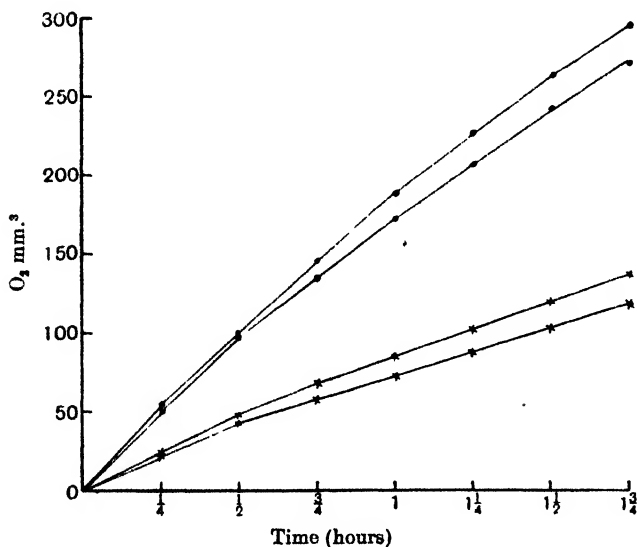


Fig. 2. O_2 consumption of *B. coli* (0.36 mg. N) + formate in presence of air (upper curves) and a mixture containing 3.88 volumes of CO to 1 volume of O_2 (lower curves). p_H 6.3, 16° .

ably further products of oxidation, continue to accumulate until, after an hour or so, a steady state is reached. At 40° the final steady state is reached in the first half hour or less, and no initial slow period is obvious.

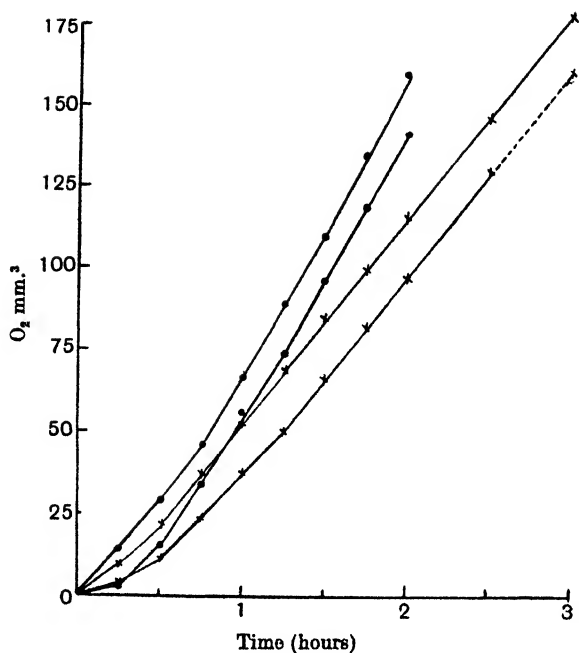


Fig. 3. O_2 consumption of *B. coli* (1.43 mg. N) + glucose in presence of air (upper curves) and a mixture containing 8.83 volumes of CO to 1 volume of O_2 (lower curves). p_H 7.6, 16° . Over the period represented by the dotted line the cup was strongly illuminated.

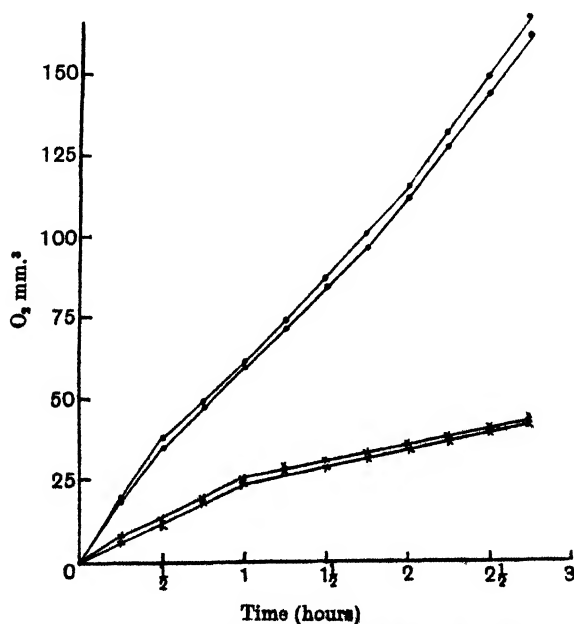


Fig. 4. O_2 consumption of *B. coli* (0.72 mg. N) + succinate in the presence of air (upper curves) and a mixture containing 9 volumes of CO to 1 volume of O_2 (lower curves). p_H 7.6, 16° .

The following calculation may make the matter clearer. Consider a substrate oxidised with constant velocity A , giving rise to a product in concentration x , and oxidised according to Henri's equation with velocity $\frac{Bx}{x+K}$. Then if v be the total velocity,

$$v = A + \frac{Bx}{x+K}, \text{ and } \frac{dx}{dt} = A - \frac{Bx}{x+K},$$

while $x = 0$ when $t = 0$. Eliminating x , and integrating,

$$\frac{(A-B)^2 t}{BK} = \frac{(A-B)(v-A)}{B(A+B-v)} + \log_e \frac{A(A+B-v)}{B(2A-v)}.$$

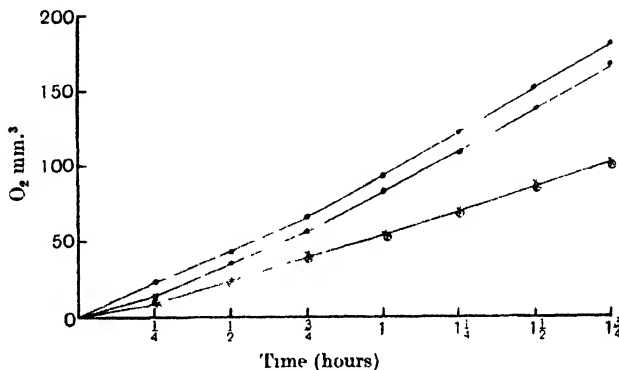


Fig. 5. O_2 consumption of *B. coli* (0.72 mg. N) + lactate in presence of air (upper curves) and a mixture containing 9 volumes of CO to 1 volume of O_2 . p_H 7.6, 16°.

Hence if $A < B$, the final value of B is $A + B$, the concentration x of the intermediate increasing indefinitely. If $B > A$, the final value of v is $2A$, the final value of x being $\frac{AK}{B-A}$. In the cases here treated it appears that $A < B$. A similar calculation can be made if there are more than two steps in the oxidation.

We find that in the oxidation of glucose and lactate it is impossible to measure the initial velocity A with any great accuracy. Results are generally irregular in the first half hour, and the velocity is already increasing at the end of an hour. On the other hand with succinate no increase occurs until nearly two hours have elapsed. This is explained by the fact that fumarate, the immediate product of oxidation, must be hydrated to malate by fumarase before it can be further oxidised. There must be an accumulation, first of fumarate, then of malate. Only when enough of the latter has been formed will the rate of oxygen-uptake increase appreciably. In each case the final rate of oxidation exceeds the initial by 30-40 %.

Comparative rates of oxidation of different substrates.

Table I gives the final rates reached after two hours by the organism when washed free of substrate, and in presence of the four substrates employed. The results are expressed in $mm.^3 O_2$ (reduced to 0° and 760 mm.) per mg.

Table I.

Substrate	None	Glucose	Succinate	Lactate	Formate
	16 (p_H 7.6)	72	95	158	472
	20 (p_H 6.3)	69	116	140	486
				131	
Average	18	71	106	143	479
Q_{O_2}	-217	-855	-1277	-1723	-5771

bacterial N per hour, and refer to a culture which had been kept for some weeks at 0° after washing. As these bacteria contain about 8.3 % of their dry weight of N, Warburg's Q_{O_2} is obtained on dividing by -0.083. It will be seen that these values are very much larger than those found with yeast or mammalian tissues, where Q_{O_2} values of -60 and -20 respectively are normal. The contrast would have been still greater had our experiments, like most on yeast and tissues, been performed at 25° or 37°, instead of 16°. With fresh bacteria the rates of glucose and lactate oxidation are about the same. Thus in two experiments, one three days after the other, with a fresh suspension, we found O_2 -uptakes per hour per mg. N of 162 and 124 mm.³ in presence of glucose, 170 and 126 in presence of lactate. It would seem probable that the enzymes concerned in the breakdown of glucose to more readily oxidised substances are less stable than the oxidising enzymes.

Some light is thrown on the nature of these products by experiments on fresh suspensions where glucose and lactate were oxidised alone and in mixture. The rates of O_2 consumption in mm.³ were:

Glucose	Lactate	Sum of rates	Glucose-lactate mixture
172.5	180.6	353.1	272.9
132.0	134.4	266.4	195.2

It will be seen that the oxygen consumption in presence of the mixture was 77 % and 73 % respectively of the sum of the rates in presence of glucose and lactate alone. On the other hand Quastel and Wooldridge [1927] found that the rate of methylene blue reduction by a glucose-lactate mixture was, if anything, slower than by lactate alone, which, as in our case, was a speedier reducer than glucose. The most obvious interpretation of our results is that some, but not all, of the glucose is transformed into lactic acid before oxidation. As the lactic dehydrogenase is already saturated with its substrate, the transformation of glucose into lactic acid does not increase the oxidation rate of the mixture. But about half the glucose appears to be converted into another substance (perhaps methylglyoxal) or substances, which are oxidised by different enzymes. This fraction cannot be calculated exactly, because substances may unite with enzymes which cannot activate them, thus inhibiting the oxidation of the proper substrate. It is conceivable that glucose may slow down the action of lactic dehydrogenase in this way. A comparison of our results with those of Quastel and Wooldridge suggests that the intermediary substances other than lactic acid reduce oxygen more readily than methylene blue in presence of *B. coli*.

Oxidations in presence of CO.

The oxygen-uptake of the starving organism during its first hour at 16° is considerably inhibited by the presence of 9 volumes of CO per volume of O₂ (Fig. 1). But the subsequent slow oxygen-uptake is hardly affected even when the ratio is raised to 15:2. Actually the duplicate uptakes in an hour without CO were 62.6 and 61.7 mm.³, and with CO 57.5 and 57.6, giving a value of 190 for K, the apparent ratio of the affinities for CO and O₂. This agrees with Warburg's [1928] results with starved yeast, whose O₂ consumption is unaffected by CO.

Cook, Haldane and Mapson found that the toluene-treated bacteria gave very different values for K (the ratio of CO to O₂ giving 50 % inhibition) according to the substrate oxidised. Comparable results have been obtained with the untreated organism and are given in Table II.

Table II.

Substrate	K (toluene)	K (no toluene)
Glucose	—	19.9, 27.6
Succinate	6.3	5.8, 6.0
Lactate	9.7	9.5, 11.2, 13.7
Formate	2.3	2.4, 3.3

The method of calculation was the same as that given by Cook, Haldane and Mapson. With the exception of the value of 11.2 for lactate, all the above figures have a considerable probable error owing to inconstancy of oxidation rates, and occasionally to disagreement of duplicates. Little difference was found between the values during the initial phase of oxidation and the final, more rapid, phase. Thus the figure 19.9 for glucose is the mean of 19.0, obtained from the readings between 30 and 60 minutes from the beginning of the experiment, and 20.8, obtained from the readings between 90 and 210 minutes, over which period the rate of oxygen-uptake was nearly constant. It is clear that the values of K are, within the limits of experimental error, unaffected by toluene treatment. The oxidation of glucose is clearly less sensitive to CO than that of the other substrates, the small inhibitions obtained rendering an exact evaluation of K impossible. The slight inhibition is explained if the glucose is largely converted into lactic acid before oxidation. As the system is not saturated with lactate, the effect of CO is lessened, for, as Warburg points out, it is no longer the limiting factor.

Just as in the case of the toluene-treated organism, the inhibition by CO was practically unaffected by illumination with a fairly powerful lamp.

Oxidations in presence of phenylurethane.

When the 3 cc. of suspension included 0.5 cc. of water saturated with phenylurethane, we obtained, just as with the toluene-treated organism, only a slight inhibition. This amounted to 19 % in the case of glucose. When however CO was also present, its effect was greatly augmented. The value of K for

formate was lowered from 2.9 to 0.56. In the case of the toluene-treated organism it fell from 2.3 to 0.21. The effect without toluene was therefore comparable, though less marked.

Oxidations in presence of cyanide and other inhibitors.

The widely different sensitivity to cyanide of lactate and formate oxidation found in presence of toluene is equally characteristic of the normal bacteria. In 0.001 *M* KCN the rate of oxidation of formate was reduced to 4.6 % of the normal, that of lactate to 44 %. The corresponding figures in presence of toluene are 1.6 % and 39 %. In each case the figures for formate are a little uncertain, owing to the very slow rates, which could hardly be measured exactly. The great difference in susceptibility of the two oxygenases is evident.

In view of the fact that 2-aminophenol-4-sulphonic acid forms a complex with iron, and has been used by Krah [1930] to inhibit biological reactions believed to be catalysed by that element, it was thought possible that it might inhibit oxygenase activity. However 0.5 % of this reagent only reduced the rate of oxidation of formate by 12 %, which may not have been a specific effect. This does not of course disprove the presence of iron in oxygenase, but suggests that it is firmly bound there.

It was shown by Cook, Haldane, and Mapson that methylene blue could replace oxygenase as an intermediary between the dehydrogenases of the

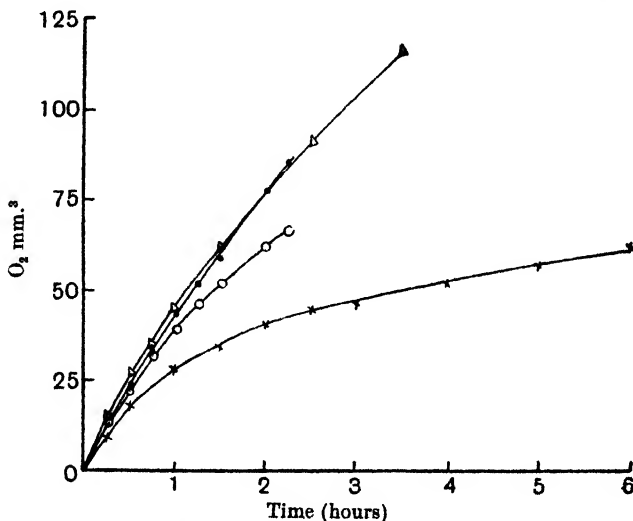


Fig. 6. O_2 consumption of *B. coli* (about 0.7 mg. N) + lactate, alone, and in mixtures containing cyanide, methylene blue and 8-hydroxyquinolinesulphonic acid (Q). p_H 7.6, 16°. Duplicate experiments agreed, but are omitted.

●—●—● Lactate control. △—△—△ Lactate + $M/1000$ KCN + $M/750$ M.b.
○—○—○ Lactate + $1/250$ Q. ×—×—× Lactate + Q + M.b. + KCN.

toluene-treated organism and oxygen, when the oxygenase had been inhibited by KCN. The autoxidation of the methylene blue, which is catalysed by a

metal, can then be prevented by CO. We find that 8-hydroxyquinoline-sulphonic acid has a similar effect. Fig. 6 shows that this substance has little effect on the oxidation of lactate. But when, in presence of cyanide, oxygenase is replaced by methylene blue, the re-oxidation of leucomethylene blue, and hence the oxygen-uptake, is greatly slowed down by 8-hydroxyquinoline-sulphonic acid, the blue colour fading considerably. In the experiment here recorded the rate fell to 14 % of that in presence of methylene blue + cyanide. 8-Hydroxyquinolinesulphonic acid unites with copper, manganese, and nickel, but not iron. Hence our experiment confirms the view of Reid [1930] that the re-oxidation of leucomethylene blue is due to copper catalysis. It may also be regarded as a "model" illustrating the experiments of Hecht and Eichholtz [1929] in which tumour glycolysis, on the ground of its inhibition by various substances, was shown probably to include a catalysis by copper.

SUMMARY.

1. When *B. coli* oxidises glucose, succinate, and lactate (but not formate) at 16° the velocity of oxidation does not reach a maximum till more than an hour has elapsed, owing to the gradual production of further metabolites.
2. The rates of oxidation, especially of formate, are much greater than those caused by yeast or mammalian tissues.
3. The effects of CO and HCN on these oxidations differ greatly, as with the toluene-treated organism, thus confirming the view that at least three different oxygenases are present in the cell.

REFERENCES.

- Cook, Haldane and Mapson (1931). *Biochem. J.* **25**, 534.
 Hecht and Eichholtz (1929). *Biochem. Z.* **206**, 282.
 Krah (1930). *Biochem. Z.* **219**, 432.
 Quastel and Wooldridge (1927). *Biochem. J.* **21**, 1224.
 Reid (1930). *Ber. deutsch. chem. Ges.* **63**, 1920.
 Warburg (1928). Über die katalytischen Wirkungen der lebendigen Substanz (Berlin).

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substitute The rates of oxidation are of the same order
 as those found for yeast and mammalian tissues.

C. A QUANTITATIVE METHOD FOR THE DETERMINATION OF VITAMIN C.

BY KATHLEEN MARY KEY AND GORDON KIRBY ELPHICK.

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(Received May 1st, 1931.)

It has been shown by Zilva and Wells [1919], Höjer [1924, 1926], Goettsch [1928] and others that a scorbutic diet will produce histological changes in the teeth of young guinea-pigs. A transverse section of the root of the incisor of a young, normal guinea-pig shows a layer of long parallel odontoblasts surrounding the pulp and separated from the regular dentine by a narrow predentine. Fine canals cross the predentine and dentine from the odontoblasts. In a scorbutic tooth the odontoblast layer is disorganised, the predentine is calcified and an irregular layer of bone is formed between the predentine and the odontoblasts. The dentine canals are fewer and are only found radiating from the predentine outwards. Höjer described ten stages in the development of scurvy based upon the amount of disorganisation of the odontoblasts, the width and irregularity of the inner dentine or layer of bone, the degree of calcification of the predentine and the structure of the dentine. He expressed the amount of protection from the disease afforded by any dose, as a fraction of complete protection, and he claimed that this fraction represented the ratio of the test dose to the minimum dose which would give complete protection. Goettsch [1928] repeated Höjer's method and found that the great individual variations of the animals made it difficult to determine the fractions of the minimum protective dose. She could, however, find a dose such that all the animals receiving it were fully protected from scurvy and those receiving lower doses were partially protected in varying degrees. She compared the potency of two substances by determining the minimum dose of each which would produce complete protection in all the guinea-pigs given that dose. The ratio of the potency of the two substances was given by the inverse ratio of the minimum protective doses. Goettsch also determined the minimum protective dose of a substance by Zilva's method in which the diagnosis of scurvy is based on growth, haemorrhages of the joints, swelling and histological appearance of the rib junctions. She found that the minimum protective dose determined by Zilva's method was approximately one-half the dose necessary in Höjer's test. The ratio of the minimum protective doses of two substances was, however, the same in both tests. In these experiments no use could be made of results given

by animals receiving doses which were less than the minimum fully protective dose. If, as appears probable from the work of Goettsch, it may be assumed that using her diet the appearance of the teeth is entirely dependent upon the amount of vitamin C present, then it follows that the degree of scurvy produced must be graded to the dose of vitamin C, provided that sufficient animals are given each dose to eliminate differences due to individual variations. If a relation could be found between the average amount of protection given by a dose and the dose, then the dose which would produce full protection could be calculated for an unknown substance. The method used by us for determining the antiscorbutic potency of graded doses of orange juice was based on the results of Höjer and Goettsch.

The oranges were bought in London and stored at a temperature of about 5°. A fresh orange was cut each day and the juice was expressed and strained through muslin before being given to the animals through a graduated pipette. The guinea-pigs were imported one dozen at a time and were kept in the laboratory for 24 hours before the beginning of each test. During that period they were given a complete diet of oats, bran and plenty of greenstuff. The first batch of oranges was bought on November 7th, 1930, and used for feeding three dozen guinea-pigs which were received in the laboratory on November 6th, 1930, December 9th, 1930, and January 1st, 1931, respectively. The second batch of oranges was bought on January 23rd, 1931, and used for feeding two dozen guinea-pigs received on January 22nd and February 12th respectively.

All the guinea-pigs used for the test were given unlimited quantities of a basal diet consisting of bran 45 %, crushed oats 25 %, and dried skimmed milk 30 %, together with 1 cc. cod-liver oil twice weekly and tap-water *ad lib*.

Each set of one dozen guinea-pigs was divided into four groups of three animals and the members of each group received no dose, and daily doses of 0.75 cc., 1.5 cc., 3.0 cc. orange juice respectively. The weight of each animal was recorded twice weekly. The test was continued for 14 days, then all the animals were killed and examined for any macroscopic lesions. Their jaw-bones were placed in Jenkin's decalcifying solution. The growth response, *post mortem* observations and structure of the roots of the incisors are detailed below.

(a) *Growth responses.*

Guinea-pigs weighing between 250 g. and 350 g. were chosen for the test. The average initial weight was 293 g. In Fig. 1 composite growth curves are shown for the animals on each dose. Good growth was obtained in each group and there was no appreciable difference between the growth of the animals receiving only the basal diet and those receiving orange juice in addition. There is no evidence to show that the slower rate of growth of the control animals during the last few days of the test was in any way due to the onset of scurvy. Great variation was found in the growth of the individuals in each group, as will be seen from Table I.

Table I.

Daily dose of orange juice cc.	Greatest increase in weight of any individual in group g.	Least increase in weight of any individual in group g.	Average increase in weight g.
No dose	75	5	38
0.75	85	- 5	50
1.5	75	20	46
3.0	80	15	48

These results show that, for a test of such short duration as 14 days, the growth of a guinea-pig cannot be taken as any indication of the antiscorbutic value of its diet.

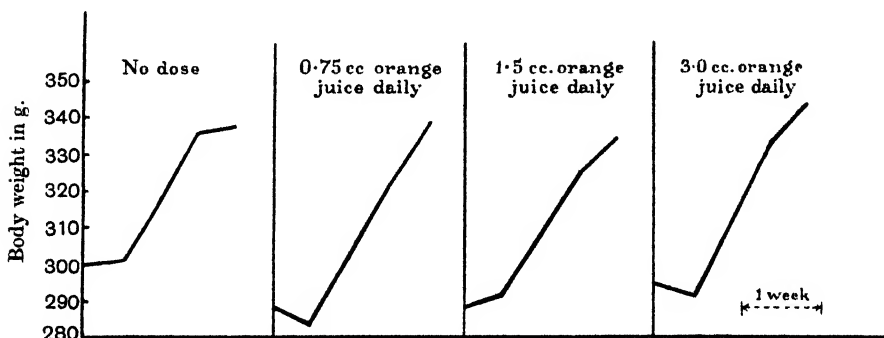


Fig. 1. Composite growth curves (average weights of 15 animals in each group).

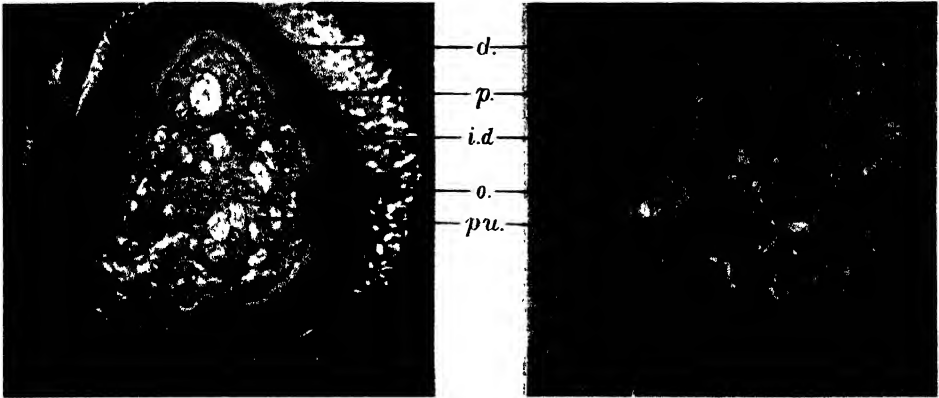
(b) Post mortem examination.

When they were killed all the 59 animals used in the experiment appeared to be in good, healthy condition except seven which had abscesses on the neck. Five others were found to have small neck abscesses when *post mortem* examinations were made. Special care was taken to note any haemorrhages at the knees or ribs and any swelling of the costochondral junctions of the ribs. These effects were, however, slight compared with those obtained in animals suffering from severe scurvy due to prolonged feeding on a deficient diet. The results are shown in Table II.

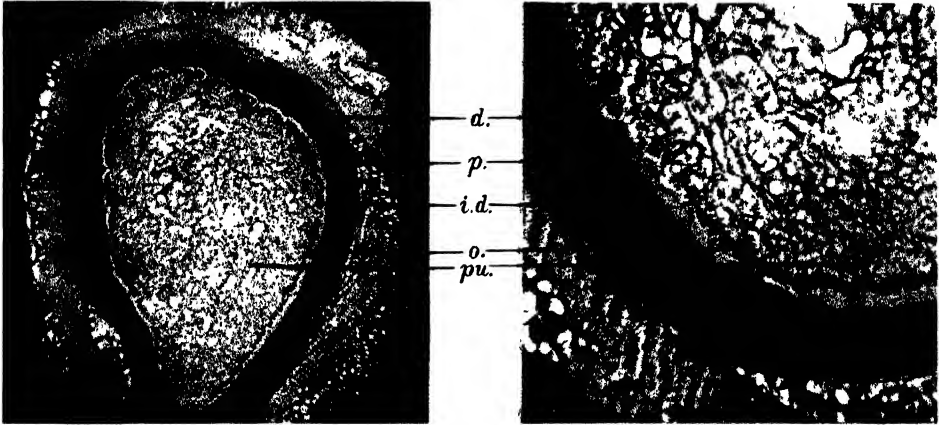
Table II.

Daily dose of orange juice cc.	No. of animals showing any haemorrhages at joints	No. of animals showing swelling of rib junctions	No. of animals with abscesses on neck	Remarks
No dose	11	2	5	3 animals had slight congestion of lungs.
0.75	6	.	2	1 animal had slight congestion of lungs. 2 animals had slight infection in stomach and intestines.
1.5	3	1	3	1 animal had slight congestion of lungs.
3.0	5	.	2	2 animals had slight congestion of lungs.

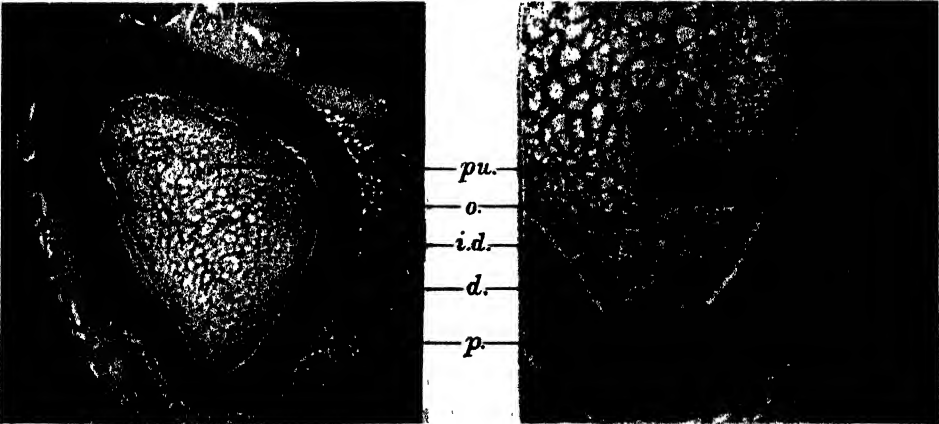




Degree of protection from scurvy 0



Degree of protection from scurvy 1



Degree of protection from scurvy 2

It will be seen that 11 of the 15 animals receiving no orange juice developed some haemorrhages at the joints. In the other groups the proportion of affected animals was lower. Similarly, two animals amongst those receiving no orange juice showed swelling of the rib junctions and five had abscesses on the neck. The corresponding figures for the other groups were lower. Thus macroscopic *post mortem* examinations showed that at the end of 14 days some difference could be detected between a scorbutic diet and a similar diet supplemented with orange juice, but no distinction could be made between the effect of different amounts of orange juice.

(c) *Structure of transverse sections of incisor roots.*

The method finally used for determining the relative potency of different doses of orange juice was based solely on the histological appearance of the roots of the incisors. In order to obtain a numerical value for the degree of scurvy produced in each animal, an arbitrary scale was devised in which the figures 0 to 4 represented stages from severe scurvy to complete protection from the disease. Plates IV and V show the characteristic appearance of transverse sections of the incisors in each group. A detailed description of the groups is given below and a summary will be found in Table III.

Group 0. In this group the odontoblasts were completely or almost completely disorganised. The inner dentine was wide, being approximately one-third the width of the dentine itself. It projected into the pulp and in some sections completely enclosed small portions of the pulp. The predentine was amorphously calcified and the Tomes's canals occurred only in the outer dentine. This picture represented the severest form of scurvy which we had produced in 14 days.

Group 1. The odontoblasts in this group were completely disorganised in some parts of the section, but in other places they were short and parallel, being only disorganised near the pulp. The inner dentine was narrower than in group 0 and although it was irregular near the odontoblasts, there were no long projections into the pulp. The predentine was calcified. In some of the animals included in this group the Tomes's canals occurred only in the outer dentine, in others a few canals could also be seen in the inner dentine.

Group 2. This group could be distinguished from the preceding ones in that the odontoblasts were all parallel though short and disorganised near the pulp. The inner dentine was narrow and regular, the predentine was either calcified or partially calcified and some Tomes's canals occurred in the inner dentine.

Group 3. The animals included in this group had only slight scurvy. The first part of the tooth to become affected by any lack of vitamin C in the diet appears to be the odontoblast layer. The cells, which are normally long and parallel, become disintegrated towards the pulp though still retaining their long and parallel formation. Teeth showing odontoblasts of this type were included in group 3 when there was no inner dentine and the predentine and Tomes's canals were normal. A few animals had teeth which were normal in

all respects except for a mere rim of inner dentine and slight disintegration of the odontoblasts. These were also included in this group.

Group 4. The animals in this group were completely protected from the onset of scurvy and consequently their teeth were normal. The odontoblasts were long and parallel. There was no inner dentine. The predentine was not calcified and the Tomes's canals crossed the dentine from the odontoblasts.

It was found that nearly all the teeth examined would fit into one of these five groups. There were a few exceptional animals in which one part of the tooth would indicate severe scurvy and justify inclusion in group 0, while another part of the section conformed with group 3 or 4. For example, a wide inner dentine was sometimes found accompanied by long, parallel odontoblasts, only slightly disorganised near the pulp. Such sections were judged separately by two individual workers and an average value for the degree of protection from scurvy was determined. In two teeth a series of two or three calcified rings could be seen in the dentine, resembling a series of calcified predentines. We could not understand the origin and interpretation of this structure.

In a normal tooth the dentine is often very wide, even approximately equal in thickness to the widest part of the pulp. In a scorbutic tooth the dentine is comparatively narrow, but since the width depends to some extent upon the exact position of the transverse section, this feature cannot be used as a criterion for determining the degree of scurvy.

Each guinea-pig used in the test was assigned a value for the degree of protection from scurvy in accordance with the scheme which is summarised in Table III. The results are shown in Table IV.

Table III.

Odontoblasts	Inner dentine	Predentine	Tomes's canals	Degree of protection from scurvy
Disorganised	Wide, with projections into pulp	Calcified	Only in outer dentine	0
Completely disorganised in places but some parallel formation	Narrow and irregular	Calcified	Only in outer dentine or mostly in outer dentine	1
All parallel but becoming disorganised near pulp	Narrow	Calcified or partially calcified	Mostly in outer dentine or crossing inner and outer dentine from odontoblasts	2
Long and parallel but becoming disorganised near pulp	Absent or mere rim	Not calcified	Cross dentine from odontoblasts	3
Long and parallel	Absent	Not calcified	Cross dentine from odontoblasts	4

The teeth of all the animals which received no orange juice showed severe or moderately severe scurvy and the degree of protection was estimated as

Table IV.

No dose		0.75 cc. orange juice		1.5 cc. orange juice		3.0 cc. orange juice	
Guinea-pig	Degree of protection from scurvy	Guinea-pig	Degree of protection from scurvy	Guinea-pig	Degree of protection from scurvy	Guinea-pig	Degree of protection from scurvy
1	0	4	0	7	3	10	Died on 4th day of test
2	0	5	2	8	1	11	4
3	0	6	1	9	0	12	3
13	0	16	2	19	3	22	4
14	0	17	2	20	3	23	4
15	0	18	2	21	3	24	4
25	1	28	0	31	2	34	3.5
26	1	29	1	32	3	35	3.5
27	1	30	1	33	2	36	4
37	1	40	1	43	3	46	4
38	1	41	2	44	4	47	4
39	1	42	1	45	2	48	4
49	1	52	2.5	55	3	58	4
50	1	53	3	56	1	59	4
51	1	54	1	57	3	60	4
Average value		0.6	1.4	2.4	3.9		

0 or 1 according to the scheme in Table III. 3.0 cc. orange juice was the highest dose tested and this produced complete protection in 11 of the 14 animals used, and nearly complete protection in the remaining three. The effect of the lower doses of orange juice was more irregular. For example, among the 15 guinea-pigs given a daily dose of 1.5 cc. orange juice, one was assigned the value 0 and one the value 4, the remaining animals being given intermediate figures. It was assumed that the average value for the 15 animals receiving each dose represented the true protective power for that dose as measured by our arbitrary scheme. The average value for the degree of protection from scurvy was plotted against the dose of orange juice and a straight line was obtained as shown in Fig. 2.

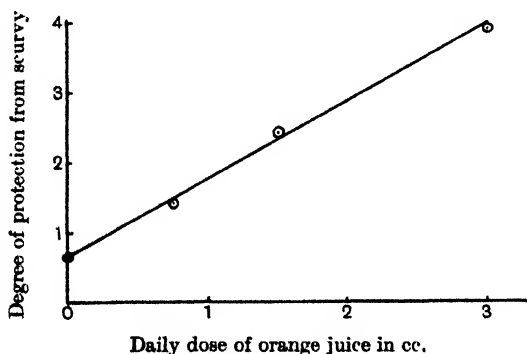


Fig. 2.

This curve could be used to compare any unknown substance with any standard. Suppose, for example, the potency of the unknown were required in

terms of decitrated lemon juice¹. Some guinea-pigs would be given the unknown and others the lemon juice. The average degree of protection given by each dose of unknown and by each dose of lemon juice would be determined. Suppose the average degree of protection given by 1.5 cc. lemon juice were 2.4 and that given by a similar dose of unknown, 1.6, then the relation between the lemon juice and the unknown would be given by the ratio of the corresponding abscissae obtained from Fig. 2. Thus

$$\frac{\text{Potency of unknown}}{\text{Potency of lemon juice}} = \frac{0.85}{1.5} = 0.6.$$

Orange juice compared with lemon juice as standard.

Orange juice is useful as a standard of reference for vitamin C since it is easily obtained and can be given directly to the animals without previous treatment. The guinea-pigs like the juice and drink it readily. Lemon juice is unpalatable unless decitrated and the process of decitration is difficult to control on the small scale. If insufficient calcium carbonate is added, a cloudy filtrate is obtained even after standing for two or three hours. If too much calcium carbonate is added, very little juice is obtained. Different lemons require different amounts of calcium carbonate for the best yield of decitrated juice. It is a disadvantage that a standard should require preliminary treatment of this sort, which differs for different samples of lemon juice. Orange juice, is, therefore, more convenient as a standard if it may be assumed that its potency is equally constant. In our experiments no difference in activity has been detected between the fresh oranges and those which had been stored for three months at 5°. Also there appeared to be no seasonal variation in the response of the guinea-pigs during the four months November to February. These results were, however, inconclusive and require further investigation. During the period from October 1928 to August 1930, 28 guinea-pigs were given a daily dose of 3 cc. orange juice. They were included in eight different experiments at different times of the year. All the animals were fully protected from scurvy except one which showed slight symptoms of the disease. It appears from these results that the antiscorbutic potency of orange juice is probably just as constant for different oranges as is the antiscorbutic potency of lemon juice.

Determination of the antiscorbutic potency of an unknown substance in terms of the potency of orange juice.

The dose of orange juice whose antiscorbutic activity is equal to that of a given dose of unknown may be determined from Fig. 2. The average value for the degree of protection from scurvy given by the unknown is found by experiment and the corresponding dose of orange juice is obtained directly from the curve.

¹ The use of lemon or orange juice as a standard of antiscorbutic potency has been provisionally recommended by a Sub-committee of the Committee on Accessory Food Factors appointed by the Medical Research Council and Lister Institute.

Exp. 1. In a test of Bramley's seedling apples in which three animals were used for each dose the following results were obtained:

Daily dose of apple = A g.	Degree of protection from scurvy shown by each animal on dose	Average degree of protection for each dose	Corresponding dose of orange juice = B (From Fig. 2) cc.	Dose of orange juice equivalent to 1 g. apple = B/A cc.
1.5	1 0 1	0.67	—	—
3.0	1 0.5 2	1.17	0.5	0.17
6.0	4 3 0	2.33	1.47	0.24

Thus the degrees of protection produced in the three animals receiving 3 g. apple were 1, 0.5 and 2 respectively. The average of these is 1.17. From the curve in Fig. 2 it will be seen that the abscissa corresponding with 1.17 degrees of protection gives 0.5 cc. as the equivalent dose of orange juice. The vitamin C content of 3 g. apple is, therefore, equal to that of 0.5 cc. orange juice and the dose of orange juice equal to 1 g. apple is 0.5/3 or 0.17 cc. Similarly 6 g. apple were found to be equivalent to 1.4 cc. orange juice, therefore, 1 g. apple is equivalent to 0.24 cc. orange juice.

Thus the antiscorbutic potency of 1 g. apple is approximately equal to that of 0.2 cc. orange juice. Greater accuracy could be obtained by the use of more animals on each dose. The error caused by individual variations must be very great when one animal out of the three receiving 6 g. apple gave the value 0 and another gave the value 4. In the following experiment five animals were used for each dose.

Exp. II. A sample of diluted tomato juice of unknown strength was examined for its vitamin C content. Daily doses of 3 cc., 6 cc., and 12 cc. were chosen and five guinea-pigs were used for each dose. The results were estimated independently by two experienced and two inexperienced observers. The results are shown in Table V.

Table V.

Dose of diluted tomato juice = A cc.	No. of guinea-pig	Values assigned by				Average for each animal	Average for each dose	Corresponding dose of orange juice (from Fig. 2) = B cc.	Dose of orange juice equivalent to 1 cc. tomato juice = B/A cc.
		KK	GE	FD*	BM*				
3	61	4	4	4	4	4.0	1.4	0.7	0.23
	62	2	1.5	2	1	1.6			
	63	0.5	0	1	1	0.6			
	64	0	0	0.5	0.5	0.2			
	65	0.5	0	1	0	0.4			
6	66	3	3	3	2	2.7	2.2	1.4	0.23
	67	0	0	0	0	0			
	68	4	4	4	3	3.7			
	69	3	3	3	2	2.7			
	70	2	1.5	2	2	1.9			
12	71	4	4	4	4	4.0	3.7	2.7	0.225
	72	4	4	3	4	3.7			
	73	3	3	3	3	3.0			
	74	4	4	3.5	4	3.9			
	75	4	4	3.5	3.5	3.7			

* Observers with very little experience of normal and scorbutic teeth. Their estimates were given entirely from Plates IV and V and Table III.

It will be seen that the independent observations were in close agreement and that the three doses gave the same value for the potency of the tomato juice. This result is probably less accurate than the figures appear to indicate but the experiment shows that when five animals are given each dose, the differences due to individual variations are to a large extent eliminated.

DISCUSSION.

The method described in this paper shows that graded responses can be obtained with different doses of vitamin C when the average protective value is determined for each dose. The curve relating the response to the dose may be used for determining the potency of an unknown substance in terms of any standard. This result is more accurate than that obtained from the minimum protective doses as recommended by Goettsch. Suppose, for example, an unknown fruit juice were examined for its vitamin C content. In order to determine the minimum protective dose, it would be necessary to test a wide range of doses, 0.5 cc., 1 cc., 2 cc., 4 cc. and 8 cc. might be suitable daily doses. Suppose three animals were used for each dose and all those receiving 4 cc. and 8 cc. were fully protected from scurvy while those receiving 2 cc. were not fully protected. Then the minimum protective dose of the unknown is less than 4 cc. but more than 2 cc. Since the minimum protective dose of orange juice is 3 cc., the ratio

$\frac{\text{Potency of unknown}}{\text{Potency of orange juice}}$ is greater than $\frac{2}{3}$ or 0.75 and less than $\frac{4}{3}$ or 1.5.

If the same fruit juice were examined by the method which is here described, fewer doses would be required though a wide range would still be necessary in order to obtain at least one dose which would give partial protection. Suppose the fifteen animals were divided into three groups of five individuals and the doses chosen for each group were 0.5 cc., 2 cc., and 8 cc. respectively. The following results might be obtained:

Daily dose of unknown = A cc.	Average degree of protection	Corresponding dose of orange juice = B (From Fig. 2) cc.	Dose of orange juice equivalent to 1 cc. of unknown = B/A cc.
0.5	1.3	0.6	1.2
2.0	2.9	2.0	1.0
8.0	4.0	Not < 3.0	Not < 0.4

Obviously 8 cc. is more than enough to produce complete protection. It is therefore more potent than 3 cc. orange juice. No further information can be gained from this dose. The lower doses show that the ratio

$\frac{\text{Potency of unknown}}{\text{Potency of orange juice}}$ equals approximately 1.1

since this is the average of the values in the right-hand column. This result agrees with that obtained by the minimum protective dose method but gives a more precise result.

Similar results could be obtained with decitrated lemon juice or any other standard.

This method is particularly useful for determining the antiscorbutic potency of substances containing little vitamin C. It is often difficult to give guinea-pigs high enough doses of such substances to produce complete protection from scurvy so that the minimum protective dose cannot be directly determined. Also when testing an unknown substance, a dose might be chosen which would produce partial but not complete protection. The corresponding dose of orange juice could be directly determined from the curve in Fig. 2.

SUMMARY.

A method is described for determining the antiscorbutic potency of a substance in terms of the potency of a standard such as lemon juice or orange juice. Different doses of orange juice were examined on a series of guinea-pigs and the amount of protection from scurvy produced in each animal was determined by means of an arbitrary scale. A curve was constructed relating the average amount of protection afforded to the dose of orange juice given. A straight line was obtained and this was used as a curve of reference for evaluating results on an unknown substance. Some disadvantages of the use of decitrated lemon juice as a standard are pointed out, and evidence is given which suggests that the antiscorbutic potency of orange juice is constant.

REFERENCES.

- Goettsch (1928). *Quart. J. Pharm.* **1**, 168.
Hojer (1924). *Acta Paediat.* **3** (suppl.).
— (1926). *Brit. J. Exp. Path.* **7**, 356.
Zilva and Wells (1919). *Proc. Roy. Soc. Lond.* B **90**, 505.

DESCRIPTION OF PLATES IV AND V.

PLATE IV. *d.* dentine showing Tomes's canals. *p.* calcified predentine. *i.d.* inner dentine. *o.* odontoblasts. *pu.* pulp.

PLATE V. *d.* dentine showing Tomes's canals. *p.* uncalcified predentine showing Tomes's canals. *i.d.* inner dentine. *o.* odontoblasts. *pu.* pulp.

CI. THE ACTION OF DYESTUFFS ON ENZYMES.

II. FUMARASE.

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It has been shown in an earlier communication [Quastel and Wheatley, 1931] that there is a marked difference of behaviour between the various dyestuffs in their effects upon the oxidations of bacteria and tissues. Whereas certain dyestuffs such as ethyl violet or malachite green will eliminate almost entirely the ability of bacteria to oxidise such substrates as glucose, succinate, lactate or formate, others, *e.g.* Congo red, crystal scarlet, are inert. It is among the basic dyestuffs that the greatest toxicity is discernible; the acid dyestuffs appear to be comparatively inactive under the conditions employed. Not only, however, does the basic nature of the molecule play an important part in determining its toxic action; its molecular structure is of equal importance. It has been shown, too, that toxicity of a dyestuff towards oxidations is greatly influenced by the presence of substances which appear to have little to do with the oxidations themselves. Methylene blue, for instance, is much less toxic to the oxidation of succinate by bacteria or muscle in the presence of phosphates than in the presence of glycine or veronal at the same p_H , and it can be shown that bacteria 'poisoned' by methylene blue in the presence of veronal and unable to take up oxygen, will once again respire on the addition of phosphates. Phosphates appear to be able to combine with the dyestuff and to elute it from its combination with the oxidising system.

It is now proposed to describe the effects of dyestuffs on an enzyme, fumarase, which has a wide biological distribution. It is found in considerable amount in yeast, most forms of bacteria, red blood corpuscles, moulds, certain parts of plants and most animal tissues. The enzyme is entirely intracellular and it is owing to this fact and the circumstance that certain cells are not freely permeable to the fumarate molecule, that it is easy to make a wrong estimate of the total quantity of fumarase in the cell. Fumarase has been chosen for study not only because of the relative simplicity of the easily reversible hydrolytic reaction which it brings about but also because the experimental technique involved is simple and the effects of dyestuffs upon it can be determined with considerable accuracy.

Dyestuffs show a specificity of behaviour towards fumarase which is much

greater than that displayed towards the oxidations already referred to. Both basic and acid dyestuffs have toxic effects on the enzyme, a specificity of behaviour with both types of dyestuff being observable. The specificity, however, among the acid dyestuffs, is most marked—whereas, for instance, two such acid dyestuffs as crystal scarlet and orange G have no inhibiting action. Congo red is toxic at a molar concentration of 1.2×10^{-8} .

Preparation of fumarase.

(a) *From B. coli.* It was shown in the first place by Quastel and Whetham [1924] that fumarase occurred in the cells of *B. coli*. Its action, however, was masked by the presence of reducing systems in the cell. When these systems were inactivated by growth inhibitors such as toluene or propyl alcohol the enzyme could be demonstrated in relatively large quantity [Woolf, 1929]. As will be shown later, however, it is necessary, in order to study the effects of certain substances on fumarase, to have a preparation of the enzyme free from the cell. This preparation can be made in the case of *B. coli* in the following way.

B. coli is grown in tryptic broth or on nutrient agar plates for 48 hours and the organism centrifuged from the broth or scraped from the plates with the addition of a little normal saline. It is washed twice with saline to remove extraneous material as far as possible and finally made up into a thick suspension with saline. To this suspension is added four times its volume of 1 % Na_2HPO_4 , and this is allowed to stand in the incubator at 37° for three days. The suspension is now centrifuged and the centrifugate, which is slightly opalescent, mixed with a little kieselguhr and filtered. The clear filtrate contains the enzyme. It should be neutralised with a little acid and stored at 0° . (This preparation not only contains fumarase, but it has the property of transforming fumarate into *l*-aspartate in the presence of ammonium ions.)

(b) *From M. lysodeikticus.* If a washed suspension of this organism (prepared from a growth on agar plates) be mixed with fumarate in phosphate buffer and allowed to stand at 45° for six hours, little or no transformation into *l*-malate occurs. There would appear to be little or no fumarase present. If, however, as was shown by Penrose and Quastel [1930], the suspension of the organism be first lysed by the addition of saliva or a little egg-white, a rapid transformation of fumarate into *l*-malate takes place, the breakdown of the cell membrane bringing about a ready access of fumarate to the enzyme. Neither saliva nor egg-white enhances the activity of the cell-free enzyme. A method, therefore, of obtaining fumarase free from the cell is to lyse *M. lysodeikticus*, to centrifuge the solution after lysis and to filter the centrifugate after admixture with a little kieselguhr. The clear filtrate contains the enzyme, and more active preparations can be made in this way than by the method described for *B. coli*. The method, moreover, is far quicker, for the lysis of the organism takes place in a few minutes at 37° or 45° . The preparation is

practically free from phosphates and is convenient for the study of the actions of phosphates and salts on the enzyme.

(c) *From red blood corpuscles.* Clutterbuck [1928] reported that defibrinated ox blood showed a slight fumarase activity. This, I have found to be also the case with human, rabbit, or guinea-pig blood. Incubation of whole blood or a washed suspension of blood corpuscles with fumarate at 45° for several hours results only in a slight transformation into *l*-malate. If, however, the red blood cells be first lysed with distilled water then, just as in the case of the lysis of *M. lysodeikticus*, there is a most rapid transformation of fumarate into *l*-malate. Human blood proves, indeed, to be one of the richest sources of fumarase. (Further details of this, together with other facts concerning the biological distribution of fumarase, will be reserved for another communication.) The fumarase obtained from blood is accompanied by much protein, and this greatly affects results carried out with dyestuffs, etc.

(d) *From brain.* In estimating the effects of dyestuffs, etc. on fumarase it is of great importance to have a preparation of the enzyme which is reasonably free from protein and phosphates and which can be kept, as a standard preparation, for several months without losing its activity. None of the methods described above is particularly suitable. To obtain fumarase from bacteria involves the tedious procedure of preparing growths of the organisms, with subsequent lysis or autolysis, and the activities of the preparations are somewhat variable, depending upon the strain of the organism, effectiveness of lysis, etc.

Attempts were made to make a standard preparation from muscle but, although very active extracts were made, it was difficult to obtain a reasonably protein-free preparation which would keep for any length of time.

Finally the problem was solved by preparing the enzyme from brain. It is known that the enzyme occurs in brain, but perhaps it is not so well known that the white matter of brain is even richer in the enzyme than the grey. The whole brain, therefore, was used for the following preparation of fumarase.

The brain of an animal (I have used both sheep and human brain) is freed from the membranes and minced twice. To the minced brain is added twice its volume of 1 % Na_2HPO_4 , and the suspension is well shaken mechanically for an hour. The resultant creamy liquid is filtered through muslin, and an equal volume of saturated ammonium sulphate solution is added and the suspension well stirred and allowed to stand overnight. A firm clot of protein, rich in fumarase, appears at the surface. This is poured on to muslin, which retains the clot. The clot in the muslin is allowed to drain for 24 hours and can be squeezed to facilitate the draining. Finally it is rapidly washed with water. (This clot may be suspended in water, which takes much of it into solution, and reprecipitated with saturated ammonium sulphate—the operation described above being repeated.)

The clot, which is of slimy consistency, is spread evenly over a glass plate and about twice its weight of plaster of Paris mixed with it. An even mixing

should be made so that the heat evolved, as the preparation dries, is quickly dissipated. Usually the preparation as a whole, if properly mixed and broken, does not become more than just warm—the temperature should not be allowed to become high enough to cause destruction of the enzyme.

The plaster of Paris preparation takes up water rapidly and should be stored in a refrigerator kept at 0° or slightly below 0°. I have found that this preparation will retain its fumarase activity for six months.

20 g. of the preparation is mixed with 100 cc. distilled water and well shaken in a machine for an hour. This is centrifuged and the slightly opalescent centrifugate is treated with a few cc. of $M/2$ potassium oxalate solution to precipitate all the calcium present. About 5 g. of kieselguhr are added to the suspension, which is shaken by hand for a few minutes and filtered. A perfectly clear filtrate with high fumarase activity is formed—this contains only slight traces of phosphate and protein. It is important, when eluting the enzyme from the plaster of Paris preparation, to use distilled water and not saline or phosphate or bicarbonate solutions. If the latter are used, preparations richer in fumarase are formed, but large quantities of protein are also taken into solution.

Fumarase preparations made in this way have remarkably constant fumarase activities, fresh extracts made every two or three days over a period of several weeks not varying in activity more than 5 or 6 %.

The plaster of Paris preparation may be extracted in a Soxhlet with ether without appreciably diminishing its fumarase activity—I have found, however, for my purposes, that the ether extraction is unnecessary.

This plaster of Paris preparation will be referred to in the remainder of this paper as the brain preparation.

Estimation of fumarase activity.

The estimation of *l*-malic acid may be easily accomplished by means of the polarimeter. Malic acid gives in acid solution and in the presence of molybdate a complex ion having very high rotatory power. Full details of the molybdate method of estimating *l*-malic acid are given by Auerbach and Kruger [1923]. These workers also describe the influence of various (not polarimetrically active) substances on the malic-molybdate complex. Citrates, for example, should not be present as they have the effect of greatly increasing the rotation. (This fact was not taken into account in the work of Mann and Woolf [1930] on the effects of citrates on fumarase activity and, in consequence, it is very difficult to interpret their results.)

I have made it a routine to take 5 cc. of the solution containing the *l*-malate, to add 1 cc. glacial acetic acid and 10 cc. of 14.2 % ammonium molybdate solution [see also Woolf, 1929], to filter and examine the solution polarimetrically in either a 1 dm. or a 2 dm. tube, using the mercury green line. When certain bacteria are being used, filtration after adding acetic acid and molybdate does not always give a clear solution—it is necessary then to centrifuge the solutions.

When using dyestuffs, the main difficulty is to remove the dyestuffs without affecting the content of *l*-malate. This, however, is easily accomplished, for all the dyes I have used, by shaking the solution, after addition of acetic acid and molybdate, with about 0.25 g. of decolorising charcoal. On filtering, a clear solution is obtained which is free from the dyestuff. Under these conditions the charcoal has an inappreciable effect on the *l*-malate content.

In the experiments to be recorded the enzyme is placed in presence of 0.08 *M* solution of sodium fumarate and incubation allowed to proceed at 45°. The rate of transformation of the fumarate into *l*-malate is linear for about 50 % of its transformation into the equilibrium concentration of *l*-malate. (Equilibrium is established at a ratio of about 1 fumarate to 3 *l*-malate.)

Using a 1 dm. tube, the rotation given, under the conditions specified, by a 0.08 *M* solution of fumarate which has reached equilibrium in the presence of fumarase at 45°, is 2.32°. The transformation of fumarate into *l*-malate is linear until the rotation begins to exceed 1.2°. Hence for studying the effects of various dyes, *etc.*, on fumarase, an amount of enzyme was taken, which would give in the time of experiment, and in the presence of 0.08 *M* fumarate, a rotation of about 1.2°.

2 cc. of the brain extract, prepared as described above, was found to give in the presence of 0.08 *M* fumarate and *M*/25 phosphate buffer, p_H 7.4, a rotation of 1.0° in 2 hours at 45°. The activity of the fumarase in the extract, after submitting it to the action of dyes, *etc.*, could be easily estimated, therefore, by determining the rotation under these experimental conditions. A rotation, for instance, of 0.5° would mean that an inhibition of 50 % of the activity of the enzyme had occurred.

Effects of dyestuffs on fumarase from brain.

Table I gives a representative series of results illustrating the action of dyestuffs at a fairly high concentration (1/2000) on the fumarase in brain

Table I. *Effects of dyestuffs (1/2000) on fumarase from brain in the presence of phosphate buffer, p_H 7.4, at 45°.*

Dyestuff	% inhibition	Dyestuff	% inhibition
Congo red	100	Neutral red	0
Benzopurpurin	100	Janus green	81
Trypan blue	100	Safranine	4
Water blue	67	Methylene violet	4
Acid green	100	Bismarck brown	0
Crystal scarlet	5	Acridavine	12
Orange G	0	Eosin	51
Methyl violet 6B	100	Erythrosin	100
Ethyl violet	100	Pyronine	0
Brilliant green	0	Methylene blue	0
Malachite green	38	Toluidine blue	9

extract. The experiments were carried out as follows. In each of a series of tubes were placed 2 cc. brain extract, 1 cc. *M*/5 phosphate buffer, p_H 7.4, and 1 cc. of an aqueous solution of dyestuff. The tubes were placed in a water-bath kept at 45° for 30 minutes, and then 1 cc. of a 0.4 *M* solution of sodium

fumarate was added to each tube. They were replaced in the bath for two hours, and then 1 cc. glacial acetic acid and 10 cc. of the ammonium molybdate solution were added to each tube. The malic acid was estimated polarimetrically in a 1 dm. tube after adding a little charcoal to each solution, shaking and filtering. Table I gives the percentage inhibitions of activity effected by each dyestuff on a particular extract. It is important, as will be explained presently, when comparing the inhibitions produced by various dyestuffs to use one extract for making the comparison.

It is evident from these results that both acid and basic dyestuffs are toxic to fumarase at a concentration of 1/2000 and p_H 7.4. There is, however, a marked specificity in action both among the acid and basic dyestuffs. Thus neutral red is inert but Janus green fairly toxic; orange G is inert but Congo red highly toxic.

It is interesting to compare these results with those obtained when studying the action of dyestuffs on oxidations [Quastel and Wheatley, 1931]. Here none of the acid dyestuffs was highly toxic. Among the basic dyestuffs, acriflavine, safranine and brilliant green were highly toxic; yet these dyestuffs have no effect on fumarase under the conditions employed.

Effects of dyestuffs at various concentrations on fumarase.

It is possible to obtain a better idea of the relative toxicities of the dyestuffs by studying their action at different concentrations. Table II shows the effects of a number of dyestuffs at considerable dilution, the experiments being carried out in the manner described above and upon one extract from brain.

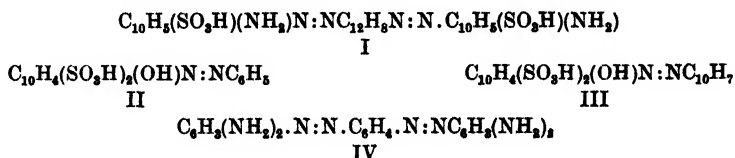
Table II. *Percentage inhibitions effected by dyestuffs at varying concentrations on fumarase (in presence of phosphate buffer, p_H 7.4, at 45°).*

Dyestuff	Concentration of dyestuff			
	1/16,000	1/32,000	1/64,000	1/128,000
Congo red	100	100	100	100
Benzopurpurin	100	100	100	100
Trypan blue	100	100	92	52
Methylene blue	12	0	0	0
Toluidine blue	10	6	0	0
Methyl violet	96	40	16	0
Ethyl violet	100	100	81	—
Crystal violet	82	21	0	0
Gentian violet	51	25	6	0
Brilliant green	7	0	0	0
Malachite green	20	10	0	0
Water blue	34	13	0	0
Neutral red	2	0	0	0
Janus green	48	8	0	0
Erythrosin	91	57	25	20

It is immediately clear that the dyestuffs most toxic at low concentrations belong to the Congo red series, *viz.* Congo red, benzopurpurin, trypan blue. Among the basic dyestuffs, those belonging to the triphenylmethane series are the most toxic, but these are not so active as the Congo red series. The acid dyestuffs of the triphenylmethane series, *e.g.* water blue, soluble blue, acid

green have a considerable toxicity but they are less active than the basic representatives. Erythrosin has a toxicity decidedly greater than that of eosin.

It is of interest to consider further the specificity of action of the dye-stuffs. Let us compare Congo red (I), orange G (II), crystal scarlet (III) and Bismarck brown (IV).



Whereas I is highly toxic, II, III and IV are inert under the conditions employed.

It is evident that the toxicity of I does not reside simply in its possession of sulphonic groups, or of amino-groups, or of the azo- or tetrazo-groups; nor is it likely that the size of the molecule is alone responsible. Possibly the benzidine nucleus is the effective group, but I have investigated the toxicity of benzidine itself and find it to be inert at a concentration of 1/1000. It would appear, therefore, that toxicity lies not in the possession of any simple grouping but on the arrangement of the molecule as a whole. The facts that benzo-purpurin and trypan blue are also toxic indicate the great importance of the spatial arrangement of the groupings within the molecules—toxicity is conferred not by any single grouping in the molecule but by the relative arrangement of a number of groupings to each other.

It is evident that the toxicity of dyestuffs to the enzyme is of much the same type as that of dyestuffs and drugs to the living cell. The fact that enzymes can be so much more easily studied than the cell as a whole should be of considerable help in elucidating the factors determining toxicity of dye-stuffs and drugs to the activities of the cell.

Effects of dyestuffs on fumarase extracted from bacteria.

The effects of dyestuffs upon fumarase prepared from *B. coli* and *M. lysodeikticus* in the manner described above are much the same as upon brain fumarase. The order of toxicities is similar. Methylene blue and toluidine blue, however, which have little toxic action on brain fumarase even at as high a concentration as 1/2000, will, at a concentration of 1/5000 entirely inhibit the fumarase prepared from *B. coli* or *M. lysodeikticus*. I have, so far, failed to discover the reason for this difference. On the whole, the fumarase from bacteria appears to be more sensitive to the action of dyestuffs than the preparation from brain. It seems probable that the differences between the extracts are due to differences in the nature of the substances accompanying the fumarase rather than to differences in the constitution of the fumarase prepared from differing biological sources. This, however, is a matter for further investigation.

Effects of dyestuffs on fumarase from blood.

The addition of dyestuffs appears to have no inhibiting action on the activity of the enzyme prepared from blood. Congo red, benzopurpurin, trypan blue, methyl violet, at concentrations as high as 1/5000 are without effect. It will be shown later that this inactivity of the dyes is due to the presence in the preparation of large quantities of protein.

Effects of the addition of mixtures of dyes to brain fumarase.

If a mixture of Congo red and methyl violet at the same concentrations be added to fumarase under the conditions described above, there is practically no toxic effect—though either of the dyestuffs alone is highly toxic at the concentration used. This result applies to many mixtures of dyestuffs. For instance, the mixtures of Congo red with crystal violet, methylene violet, methylene blue, or malachite green (where each constituent was at a concentration of 1/5000) gave percentage inhibitions of 14, 0, 5 and 6 respectively—the Congo red alone giving 100 % inhibition. On the other hand, mixtures of Congo red with water blue, crystal scarlet, or orange G were as toxic as Congo red itself.

Now it is observed that whenever a mixture of toxic dyestuffs is inert, a precipitate is formed—this being a compound or adsorption complex of the two dyestuffs. It seems that, in nearly all instances, the mixture of acid dyestuff and basic dyestuff forms a compound which is inert so far as its effect upon fumarase is concerned. On the other hand, a mixture of two acid dyestuffs or two basic dyestuffs is not less toxic than the more active of the two dyestuffs present.

The fact that acid and basic dyestuffs neutralise each other's toxic effects makes it evident that the acid or basic nature of the dyestuffs is a factor primarily involved in the combination with, or adsorption upon, the enzyme. It is clear, therefore, that the enzyme must possess acidic and basic groups (or groups bearing opposite charges), a result which is in keeping with the standpoint taken by Michaelis that an enzyme has ampholytic properties. It is significant, however, that both groups must be ionised, under the conditions of these experiments (p_H 7.4), and it follows that the active centre, responsible for the activation of fumarate, must have a zwitterion constitution.

Combination of enzyme and fumarate.

The important question now arises as to whether the fumarate molecule is itself adsorbed or combined at the basic and acidic groups which help to make up the fumarase centre. According to the active centre hypothesis; the adsorption (or combination) and the subsequent activation of fumarate at the centre will depend on the nature and spatial arrangement of the groups composing the centre. If, therefore, as the results with dyestuffs indicate, oppositely charged groups are constituents of the enzyme, and if such groups are con-

cerned with the adsorption and activation of the substrate molecule, the compound of fumarate with the enzyme should have the effect of preventing or inhibiting the combination of the dyestuff with the enzyme. Experiment entirely confirms this.

Exp. A. To 2 cc. brain extract were added 1 cc. 0.2 *M* phosphate buffer, p_H 7.4, 1 cc. water and 1 cc. 0.4 *M* sodium fumarate. After incubation at 45° for 2 hours the rotation was 1.23°.

To 2 cc. brain extract were added 1 cc. 0.2 *M* phosphate buffer, p_H 7.4, 1 cc. 1/3000 methyl violet solution and 1 cc. 0.4 *M* sodium fumarate. After incubation at 45° for 2 hours the rotation was 1.22°.

To 2 cc. brain extract were added 1 cc. 0.2 *M* phosphate buffer, p_H 7.4, and 1 cc. 1/3000 methyl violet solution. This mixture was allowed to incubate at 45° for 30 minutes, and then 1 cc. 0.4 *M* sodium fumarate was added. After incubation at 45° for 2 hours the rotation was 0.56°.

This experiment shows that incubation with methyl violet for 30 minutes prior to the addition of fumarate gave an inhibition of 54 %, whereas when the methyl violet was added together with the fumarate, no inhibition was effected.

Exp. B. The experiment was carried out as above but instead of methyl violet solution there was added 1 cc. 1/27,000 Congo red. The addition of fumarate together with the dye resulted in no inhibition of the enzyme; previous incubation for 30 minutes before the addition of the fumarate resulted in an inhibition of 53 %.

It is important, of course, in carrying out these experiments not to have too high concentrations of dyestuffs. The higher the concentration of dyestuff, the more difficult it is to observe the 'protective' action of the fumarate.

The experiments show that fumarate prevents the combination of both acid and basic dyestuffs at the enzyme. It would be expected, therefore, that the fumarate molecule is so arranged in its combination with the enzyme that it bridges both the acid and the basic groups. This arrangement

(Fig. 1) of the fumarate molecule across two oppositely charged groups provides the mechanism required to polarise the double bond, this polarisation being necessary before activation can occur [Quastel, 1926]. Adsorption of the fumarate molecule is

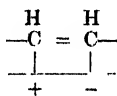


Fig. 1.

not in itself sufficient to bring about activation, for it is known that the fumarate molecule is absorbed at surfaces [Phelps, 1929], and there is no evidence that transformation to malic acid occurs. The fact, however, that the enzyme possesses two oppositely charged groups, to both of which the fumarate molecule is attached, must result in a polarising field which, in this case, is powerful enough to give the fumarate molecule the necessary energy of activation. Following polarisation, and the resulting induction of positive and negative charges at the ends of the double bond (or of an internal shift of protons and electrons), the addition of the elements of water occurs with the formation of malic acid.

It is obvious that the type of molecule which can be adsorbed at the fumarase centre, so as to bridge the two oppositely charged groups must be very limited; for, considering the dyestuffs alone, where each dye presumably attracts only one of the groups, the specificity of adsorption or combination is most marked. The fact that such specificity occurs among the dyestuffs must mean that there are important factors bearing upon adsorption or combination at the enzyme, other than simply the presence of the positively or negatively charged groups.

The question which now arises is whether the double bond is essential for the adsorption of the fumarate (or its arrangement across the oppositely charged groups) at the enzyme. Preliminary work has begun on this matter and it is clear that succinate will also 'protect' fumarase from toxic dyestuffs. It is hoped to communicate details of the investigation later.

Effects of proteins on the toxic action of dyestuffs.

The addition of protein (egg-white, serum, gelatin) to fumarase results in a very marked protective action against toxic dyestuffs.

To 2 cc. brain extract were added 1 cc. 0.2 *M* phosphate buffer, p_H 7.4, 1 cc. 1/5 human serum, and 1 cc. 1/1000 Congo red solution. This was allowed to incubate at 45° for 30 minutes, and then 1 cc. 0.4 *M* sodium fumarate solution was added. After further incubation at 45° for 2 hours the rotation was 0.75°. In the absence of Congo red the rotation was 0.96°. Serum itself has no perceptible effect on the activity of fumarase under the conditions employed.

If the serum be heated, either at 100° for a few minutes or at 55° for 30 minutes, there is a marked increase in the 'protective' action.

Results with human and rabbit serum are shown in Table III. Egg-white, gelatin and milk have similar protective actions.

Table III. *Percentage inhibitions by dyestuffs on fumarase in the presence of sera and heated sera.*

Dyestuff	Serum	% inhibition
Congo red (1/5000)	None	100
"	1/25 human serum	21
"	1/25 human serum heated at 100° for 15 min.	0
"	1/25 rabbit serum	65
"	1/25 rabbit serum heated at 100° for 15 min.	10
"	1/50 rabbit serum	80
"	1/50 rabbit serum heated at 55° for 30 min.	29
Methyl violet (1/15,000)	None	47
"	1/50 rabbit serum	0
"	1/50 rabbit serum heated at 55° for 30 min.	0

The fact that the fumarase preparation from blood is not affected by dyestuffs is clearly due to the presence of the protein.

Now it is well known that proteins greatly diminish the activity of most antiseptics, and Hirschfelder and Wright [1930] have recently shown that the

attachment of dyestuffs, *e.g.* methyl violet, to egg-albumin follows the Freundlich adsorption isotherm very closely and have adduced evidence that the reaction between the dye and the protein is a surface (adsorption) reaction and not a strictly chemical one. By dialysis experiments they were able to estimate the amount of dyestuff taken up by protein. They then studied the effects of protein in reducing the antiseptic action of dyestuffs on yeast, and found that the reduction was not as great as would be anticipated if the compound between protein and dye exerted no toxic action. They therefore concluded that the fraction of the dye which was adsorbed on the protein exerted an antiseptic action.

In a number of ways the effects of proteins on the toxic action of dyestuffs on fumarase resemble those on the antiseptic action of dyestuffs on yeast. Hirschfelder and Wright found that denatured proteins adsorb more dyestuff than native ones, and, as shown in Table III, denatured sera have a greater protective action on fumarase. The most likely explanation of the protective action of proteins on the enzyme is that the protein adsorbs or combines with the dyestuff, leaving a relatively small quantity of dyestuff free to attack the enzyme. There is no evidence from my experiments that the combination of protein and dye has any toxic action on the enzyme. It seems strange that a combination of protein and dye should exert a toxic action on the yeast cell, as Hirschfelder and Wright maintain. These workers followed the action of the dye on the CO_2 production of the cell in presence of sucrose. Presumably the action of the dye occurs within the cell and if this is so, the cell membrane must be permeable to the compound of protein and dyestuff. There is, however, the possibility, it seems to me, that the presence of the protein has an effect on the permeability of the cell, making the entrance of the dyestuff easier and hence inducing a greater antiseptic action than would have been anticipated. A point in favour of this suggestion is that the yeast produces gas more freely in the presence of protein than in its absence, pointing, possibly, to an easier access of sucrose to the enzymes of the cell. This criticism is mentioned only to indicate the difficulty of interpreting results carried out on the intact cell.

Table IV. *Effect of varying quantities of human serum on the toxic action of Congo red (1/50,000) on brain fumarase, in the presence of phosphate buffer, pH 7.4, at 45°.*

Amount of serum present cc.	Rotation observed °
None (control with no dyestuff or serum)	0.91
0.1	0.91
0.05	0.90
0.025	0.77
0.0125	0.12
0.00625	0.00

Table IV shows the effect of varying amounts of serum on the toxic action of Congo red. It will be seen that in the presence of the larger quantities of the serum, Congo red has not the least toxic action—clearly, the compound of

Congo red and protein is inert. It is therefore possible to estimate, by the fumarase method, the actual quantity of dye taken up by the protein. The method simply consists in determining that quantity of protein which, in presence of the dyestuff, brings about, say, a 50 % diminution in the activity of the enzyme. The amount of free dyestuff which accomplishes this is known and hence knowing the original quantity of dyestuff present, a subtraction gives the amount of dyestuff combined with the protein. In this manner, it may be shown that 1 cc. human serum combines with approximately 3.5 mg. Congo red.

This method of estimating the combination of protein and dye (or drug) applies, of course, only to dyestuffs (or drugs) which are toxic to fumarase—it offers, however, definite advantages over the dialysis method.

Effect of a constant amount of dyestuff on varying quantities of enzyme.

Varying quantities of brain extract were placed in a series of tubes. To each of half of these tubes was added a quantity of Congo red to make up a final concentration of 1/100,000; the other half of the tubes received no dyestuff. 1 cc. phosphate buffer, p_H 7.4, and variable quantities of water were added to each tube to make up the final volume to 8 cc. The tubes were incubated for 30 minutes at 45°, and then 2 cc. of 0.4 *M* sodium fumarate solution were added to each tube. After a final incubation for 2 hours at 45°, the rotations were determined on 5 cc. Results are shown in Table V.

Table V. *Effect of a constant quantity of Congo red (1/100,000) on varying quantities of fumarase from brain.*

Quantity of enzyme cc.	Dyestuff	Rotation observed	Inhibition	Rotation Quantity of enzyme
6	Absent	1.52°	—	0.25
6	Present	1.25	0.27°	0.21
5	Absent	1.30	—	0.26
5	Present	0.85	0.45	0.17
4	Absent	1.06	—	0.26
4	Present	0.40	0.66	0.10
3	Absent	0.80	—	0.27
3	Present	0.11	0.69	0.04
2	Absent	0.52	—	0.26
2	Present	0.00	0.52	0.00

It will be seen that, whereas in the absence of the dye the rotation is strictly proportional to the amount of enzyme, in the presence of the dye this proportionality entirely disappears.

It would have been anticipated that the greater the amount of enzyme, the greater the amount of combination between enzyme and dye and hence the greater the actual inhibition. The reverse of this occurs; the greater the amount of enzyme the less is the actual inhibition.

This result is easily explained if the assumption is made that the brain extract contains substances, other than the enzyme, which take up the dyestuff. The greater the amount of extract taken, the greater is the quantity of

impurity present which takes up dyestuff, and the smaller is the amount of the free dyestuff left over to attack the enzyme. Hence as the quantity of enzyme is decreased, the greater will be the apparent inhibition induced by the dye.

This can be placed on a mathematical basis taking the standpoint that the mass action law is followed or that the adsorption law is followed. Both laws give rise to curves which approximate closely to that found by experiment—but it is difficult from the data as yet available to decide which law is, in fact, followed.

The same phenomenon occurs with other dyestuffs and it seems to be a general rule, even with the purest extracts I have been able so far to prepare, that the actual inhibition effected by a dyestuff tends to increase as the amount of extract decreases.

It is for this reason that the comparison of the action of various dyestuffs should be carried out on one particular extract.

Comparison of effects of dyestuffs on the intact cell and on the cell extract.

When a washed *B. coli* suspension is used as a source of fumarase, the effects of the dyestuffs are, in certain instances, found to be quantitatively different from the effects on brain extract. It is shown in Table I that Congo red, benzopurpurin, methyl violet, ethyl violet and erythrosin at a concentration of 1/2000 will give 100 % inhibition of the activity of fumarase from brain. When a *B. coli* suspension is examined in precisely the same way as has been described for the extract, the following figures for the inhibitions are obtained (concentration of dyestuff, 1/2000).

Methyl violet	...	100 %	Congo red	56 %
Ethyl violet	...	100 %	Benzopurpurin	38 %
			Erythrosin	58 %

Thus, with a *B. coli* suspension the Congo red dyes are not as effective as the triphenylmethane series at these high concentrations.

When, however, an extract of fumarase is made from *B. coli* the dyestuffs show the same order of toxicity as with the brain extract.

A thick suspension of freshly grown and washed *B. coli* was incubated overnight at 37° with twice its volume of 1 % Na_2HPO_4 . It was then centrifuged and the centrifugate (or extract) separated from the deposit; the latter was made into a suspension with normal saline. Both the extract and the suspension were brought to p_{H} 7.4. The suspension of the intact cells had about twice the fumarase activity of the extract. The effects of dyestuffs on the extract and on the suspension of intact cells were then compared—amounts of extract and suspension being taken so that they had equal fumarase activity. The results are shown in Table VI. It will be seen that, whereas methyl violet and water blue had practically the same effect on the intact cell

as on the extract, Congo red and toluidine blue were decidedly less toxic to the cells.

Table VI. *Comparison of percentage inhibitions effected by dyestuffs on fumarase in the cell extract and in the intact cell (B. coli).*

Dyestuff (1/5000)	Intact cell	Cell extract
Congo red	59	97
Methyl violet	88	82
Water blue	56	57
Toluidine blue	56	90

The most probable explanation of these results is that the bacterial cell has a much greater permeability to methyl violet and water blue than to Congo red or toluidine blue.

It would seem that the *B. coli* cell is almost freely permeable to members of the triphenylmethane series but resistant to those of the Congo red series, to toluidine blue and to erythrosin.

Reversibility of the compound of enzyme and dyestuff.

Very little success has so far attended efforts to remove the dyestuff from its combination with the enzyme.

Two methods have been tried.

(1) To the mixture of enzyme and toxic dyestuff which had been allowed to incubate at 45° for 30 minutes, there was added another dyestuff, sufficient in amount to combine with the toxic dyestuff present. The effect of the second dyestuff should be to increase the activity of the enzyme by removing the first dyestuff combined with it. Congo red and methyl violet were used in such concentrations that when added together no inhibition of the enzyme was effected. On adding the methyl violet, however, after the Congo red had been in contact for 30 minutes with the enzyme, the toxic effect of the latter was not appreciably decreased—the inhibition was 92 %.

(2) To the mixture of enzyme and Congo red, after incubation at 45° for 30 minutes there was added 1 cc. serum—an amount which would protect the enzyme from a considerable quantity of the dyestuff. A slight increase in the activity of the enzyme resulted. The inhibition without the serum was 100 %; the inhibition after the subsequent addition of serum was 79 %. Clearly, a slight reversibility was effected.

It is evident from these facts that the combination between enzyme and dyestuff is very strong and offers a striking contrast to that between the enzyme and its substrate (fumarate) where an easy reversibility must occur.

Effects of phosphates on fumarase.

In view of the action of phosphates on the toxicity of dyestuffs on the oxidations of bacteria and tissues [Quastel and Wheatley, 1931] it was of interest to investigate the influence of phosphates on the effects of dyestuffs

on fumarase. Now it has been shown by Clutterbuck [1928] and later by Mann and Woolf [1930] that phosphates increase the velocity of fumarate transformation by fumarase. On making a quantitative comparison of the action of phosphates on fumarase in *B. coli* and in brain extract (containing initially less than 0.02 mg. P per cc.), it was found that the effects were not identical. Whereas phosphates at a concentration of 0.04 *M* and p_H 7.4, approximately doubled the rate of fumarate transformation in presence of *B. coli* (an initial concentration of 0.08 *M* fumarate being used), phosphates under the same conditions increased the rate of change in presence of brain extract in a ratio of 1.3. Although there is no question that phosphates increase the rate of change of fumaric acid to *l*-malic acid in presence of brain extract, the effects are apparently not as great as with intact *B. coli*. Possibly this may be due to interfering substances in the brain extract, but the criticism is justified that in work on the intact cell, as carried out by Mann and Woolf, there are also complicating factors which should be considered in drawing conclusions as to the effects of phosphates or salts on the enzyme. It seems to me, for instance, that in the work on the effects of salts, *etc.* on p_H -activity curves using the intact cells, it is not altogether certain that the interior of the cell is at the same p_H as the external medium, especially when the latter is unbuffered.

Turning now to the question of the effect of phosphates on the action of dyestuffs, it is found that a protective action exists. The protection, however, does not seem to be as great as that observed in the case of oxidations. Methylene blue and toluidine blue (1/2000), which have little or no action on brain fumarase in the presence of phosphate (0.04 *M*), exert inhibitions of 15 % and 22 % respectively in its absence. Brilliant green, however, has a very toxic action in the absence of phosphates (100 % inhibition), this being removed by the presence of phosphates. Water blue and eosin also have smaller toxic effects in the presence of phosphate than in its absence.

Whether the phosphate acts by combining with the dyestuff or whether it acts by competing with the dye for a grouping in the enzyme is a question for further investigation.

SUMMARY.

1. Cell-free preparations of fumarase have been made from bacteria, red blood corpuscles and brain. A dry preparation has been made, by the use of plaster of Paris, which retains its fumarase activity at 0° for several months. The enzyme can be eluted from the powder by distilled water and is almost free from proteins and phosphates.

2. The effects of dyestuffs and mixtures of dyestuffs on fumarase are described. Both acid and basic dyestuffs are toxic. Among the acid dyestuffs, those of the Congo red series and of the triphenylmethane series are toxic, the former being the most effective. Congo red is toxic at a concentration of 1.2×10^{-8} *M*.

3. The differences in action of dyestuffs on bacterial fumarase and brain fumarase are described.

4. Fumarate 'protects' the enzyme from toxic dyestuffs, *e.g.* Congo red or methyl violet.

5. The bearing of these facts on the constitution of the enzyme and its mode of action are discussed.

6. Proteins 'protect' fumarase from dyestuffs by combination with them, the compound of protein and dye being inert. By this means it is possible to estimate the amount of dye taken up by the protein. Denatured protein takes up more dyestuff than native protein.

7. The actual inhibition decreases as the amount of enzyme increases, when a constant amount of dyestuff is present. This is explained on the assumption that the extracts contain substances, other than fumarase, which combine with the dyestuff.

8. Differences between the intact cell (*B. coli*) and the extract from the cell are described. Permeability of the cell membrane plays an important part in determining toxicity of a dye to the enzymes of the intact cell. Whereas *B. coli* is almost freely permeable to members of the triphenylmethane series, it is resistant to Congo red, toluidine blue and erythrosin.

9. The combination of enzyme and dyestuff is not easily reversible.

10. Phosphates exert some protective action on the enzyme against toxic dyestuffs.

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REFERENCES.

- Auerbach and Kruger (1923). *Z. Unters. Nahr. Genussm.* **46**, 97.
Clutterbuck (1928). *Biochem. J.* **22**, 1193.
Hirschfelder and Wright (1930). *J. Pharm. Exp. Ther.* **38**, 411, 433.
Mann and Woolf (1930). *Biochem. J.* **24**, 427.
Penrose and Quastel (1930). *Proc. Roy. Soc. Lond. B* **107**, 168.
Phelps (1929). *J. Chem. Soc.* 1724.
Quastel (1926). *Biochem. J.* **20**, 166.
— and Wheatley (1931). *Biochem. J.* **25**, 629.
— and Whetham (1924). *Biochem. J.* **18**, 519.
Woolf (1929). *Biochem. J.* **23**, 472.

CII. THE EFFECT OF VITAMIN B DEFICIENCY UPON THE VITAMIN A RESERVES OF THE RAT.

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In a previous communication [Moore, 1931] it was shown that when rats are given diets rich in carotene, vitamin A may accumulate in the liver in amounts that seem fantastically great when compared with the minimal daily requirements of the animal. Further, if a rat which had been fed on carotene in this way were then placed upon a diet free from vitamin A the reserves of that factor did not materially decrease after a lapse of 60 days. These findings confirmed the current view that the liver possesses the power of concentrating vitamin A and storing it over prolonged periods without deterioration. In distinct contrast the fats of the other organs, together with the general storage fat of the body, continued to contain only small traces of the vitamin.

This one-sided distribution of the vitamin A reserves suggested the problem which forms the subject of the present communication. It is well known that a rat subsisting upon a diet which is deficient in the vitamin B complex loses weight progressively, and that this loss of weight is accompanied by disappearance of the body fat. In so far as the writers are aware, however, no observation has been placed on record to show whether this loss of fat is accompanied by a corresponding loss of fat-soluble vitamins. It was considered of interest, therefore, to examine the vitamin A reserves of the liver under these conditions, especially since two distinct possibilities seemed open. On the one hand, it might be supposed that in the severe emaciation resulting through vitamin B deficiency the great superfluity of vitamin A, serving no obvious purpose, would be dispersed. On the other hand, the vitamin A reserves of the liver, being concentrated there by means of some mechanism quite distinct from that regulating the decomposition and resorption of storage fat, might be expected to show no change, at least so long as the liver itself remained undamaged. This second hypothesis received *a priori* support from the observation of Drummond and Hilditch [1930] that the vitamin content of cod-liver² is not influenced by the variations in fat content associated with the sexual cycle, and in the present paper it is shown to be correct.

¹ In the whole time employment of the Medical Research Council.

² It may be pointed out that the liver of the cod may contain almost 40 % of fat, whereas in mammalian livers the fat content does not exceed about 2 %. The liver in the cod must therefore play a much more important part in the economy of stored fat than is the case with mammalian liver.

EXPERIMENTAL.

The experimental animals used were adult albino rats which had been reared to maturity upon a complete synthetic diet and subsequently maintained for a prolonged period (about 200 days) upon a diet rich in carotene ("light white casein" 20 %, rice starch 60 %, red palm oil 15 %, salt mixture 8 %, dried yeast 15 %, (additional) radiostol 1 drop per rat daily). Under these conditions the vitamin A reserves of the liver, as previously reported [Moore, 1931]¹, had been found to be remarkably constant between individuals, the usual value being about 70,000 blue units. The experimental procedure consisted of the exposure of the rats to diets deficient in the vitamin B complex until severe emaciation had resulted. The animals were then examined *post mortem* both in regard to the extent of the remaining body fat and the amount of vitamin A still contained in the liver oils.

Vitamin A reserves of the rat when fed upon a complete diet with excess of carotene. Rat 1 ♀. This animal, weight 220 g., served as a control. It was killed during the present experiment, without changing from the complete red palm oil diet, in order to ensure that the vitamin A reserves of the batch of rats used should not depart materially from the value expected through previous experience. The liver only was examined.

	Weight of fat mg.	Total natural yellow units	Total SbCl ₃ blue units
Liver	154	150	70,000

Persistence of the vitamin A reserves during continued feeding of carotene accompanied by vitamin B deficiency. Rat 2 ♂. From the complete diet containing red palm oil this rat was transferred to a diet deficient in the vitamin B complex which still contained red palm oil (Glaxo caseinogen 20 %, cane sugar 60 %, salts 5 %, red palm oil 15 %). On this diet it declined rapidly and died after 48 days, having decreased in weight from 378 g. to 171 g. *Post mortem* examination showed complete absence of fat depôts and general emaciation. Only the liver was analysed for fat and vitamin A.

	Weight g.	Weight of fat mg.	Total natural yellow units	Total SbCl ₃ blue units
Liver	6.7	114	130	110,000

Persistence of vitamin A reserves on a diet lacking both vitamin A and vitamin B. Rat 3 ♀. From the complete diet containing red palm oil this rat was transferred to a diet similar to that given to rat 2 with the exception that arachis oil now replaced the red palm oil as the fat component. The diet was therefore completely free from vitamins. The body weight fell from 266 g.

¹ The method employed in the extraction of tissues, although essentially the same as that previously described [Moore, 1930], has now been modified to permit more rapid estimations. By the addition of 50 % of alcohol to the alkaline solution of the tissue it has been found possible to remove nearly all the fat by one extraction with about 3 volumes of ether. After washing, the ethereal extract is dried by filtering through a layer of anhydrous sodium sulphate contained in a funnel provided with a sintered glass diaphragm.

to 168 g. in 35 days when the animal, being extremely emaciated, was killed. *Post mortem* examination revealed that the remains of the fat depôts were very scanty. The liver was dissected out and examined in the usual way. In addition the remainder of the carcase, including the pelt and intestinal tract and contents, was extracted with a view to finding the actual amount of fat present.

	Weight g.	Weight of fat mg.	Total SbCl ₃ blue units
Liver	6.8	120 mg.	68,000
Carcase	161	9.0 g.	Very low

Rat 4 ♀. This animal received the same diet as the preceding one but was killed after the slightly longer period of 41 days, the body weight falling from 202 g. to 126 g. At the end of this time it appeared extremely emaciated but was still active. The autopsy revealed a complete absence of intraperitoneal fat. Analyses were made separately of the liver, the intestinal tract and contents, the pelt and the remainder of the carcase.

	Weight g.	Weight of fat mg.	SbCl ₃ blue units
Liver	5.2	78	70,000
Intestinal tract	13.2	181	200*
Pelt	19.6	398	0
Carcase	85.1	114	0

* The meaning of this positive result is not clear, but faulty dissection may be suspected. This value could be accounted for by the unintentional inclusion of about 20 mg. of liver tissue.

Spectroscopic data. In the case of the liver oils examined above, the blue colour produced in the SbCl₃ reaction was characterised by the prominent band at 610–630 $\mu\mu$ which is associated with vitamin A. The natural yellow colour, when not stated, was not more than a trace. The chromogen present must therefore have consisted almost exclusively of vitamin A.

DISCUSSION.

It is at once evident from the data given that the remarkably high reserves of vitamin A which the rat is able to store up when fed on a diet rich in carotene are not depleted when it is concurrently or subsequently suffering from a lack of the vitamin B complex. The figures for rats 2, 3 and 4 show that in the state of extreme emaciation following on a deficiency of the vitamin B complex the liver oils still contained the characteristically very high vitamin A content observed by Moore [1931]. At the same time, the observation that the liver oil of rat 2 was of a normal pale colour indicates that vitamin B deficiency in no wise diminishes the power of the animal to transform carotene to vitamin A when the pigment is present in the diet in very great excess of the animal's requirements.

The data for rat 4 are particularly instructive in showing how far the loss of body fat can go without materially affecting the large vitamin A store of the liver. The total amount of fat which could be extracted from the four parts into which the body was divided was less than 1 g. The liver fat may

have been depleted (it is not quite certain whether the low figure of 78 mg. is sufficiently below the normal one of approximately 150 mg. to allow of this conclusion being drawn) but there is no doubt that the vitamin A store of the liver remained unaffected, since the figure of 70,000 blue units obtained is representative of those found for rats still receiving the complete red palm oil diet.

These results confirm the view that the vitamin A reserves of the liver constitute a system quite apart from the general fat reserves of the body. To complete the picture mention may be made of a complementary condition which is sometimes encountered in rats suffering from avitaminosis A. It is certainly true that rats surviving during long periods of decline in this condition become no less emaciated than those suffering from avitaminosis B, but when death is more sudden quite large fat deposits may remain even though no traces of vitamin A can be detected colorimetrically either in the liver or in the body fat. Thus the body of a young male rat, typical of several others, which died rather suddenly at a weight of 174 g. after 61 days' exposure to a diet deficient in vitamin A, showed almost normal fat deposits, amounting to 16.2 g., while the liver yielded 144 mg. of oil. Nevertheless, both the body fat and liver oil gave completely negative results when tested for vitamin A.

SUMMARY.

Adult albino rats, previously fed upon diets containing lavish amounts of carotene, were exposed to diets deficient in the vitamin B complex until severe emaciation had resulted. In spite of the disappearance of the fat reserves throughout the body this treatment did not lead to any material diminution in the vitamin A reserves of the liver, which remained at an extremely high level.

Our thanks are due to Dr L. J. Harris for his valuable criticism. The care of the experimental animals was in the capable hands of Mr A. Ward.

REFERENCES.

- Drummond and Hilditch (1930). The relative values of cod-liver oils from various sources (H.M. Stationery Office).
Moore (1930). *Biochem. J.* **24**, 692.
— (1931). *Biochem. J.* **25**, 275.

CIII. THE KINETICS OF PEROXIDASE ACTION.

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THIS work was started with two objects in view. First to test the suggestion of Haldane [1930], that, at low concentrations of hydrogen peroxide, peroxidase would have a temperature coefficient of unity, so that the reaction velocity would be independent of temperature, and secondly to investigate the effect of changes in the concentrations of the hydrogen peroxide and the reducing substrates on the velocity of reaction.

Willstätter and Weber [1926, 1] have shown that the temperature coefficient of peroxidase acting on leucomalachite green is $Q_{10} = 2.0$ approximately, over a range of temperature from 0 to 25°. The concentration of hydrogen peroxide was not stated but was probably 2.5 mg. per litre. Now the primary factor in the velocity of enzyme action is presumably the rate of breakdown of the enzyme-substrate compound, but Haldane suggested from kinetic considerations, that at very low hydrogen peroxide concentrations the rate of peroxidase action might rather depend on the number of collisions of hydrogen peroxide molecules with the enzyme, since most collisions would be fruitful. Since this factor is only slightly influenced by temperature, under such conditions peroxidase should have a Q_{10} of unity.

It has long been known that the velocity of peroxidase action increases with increase of peroxide concentration until an optimum is reached, after which further increase in peroxide concentration causes inhibition of the reaction. Willstätter and Weber [1926, 2] found that the inhibition thus produced by excess hydrogen peroxide was combated by increase in the concentration of the reducing substrate, such as pyrogallol or leucomalachite green. This effect has been studied in the second part of this work, using guaiacol and leucomalachite green as the reducing substrates, and from a consideration of the results obtained a new hypothesis is suggested to account for the phenomenon.

Preparation of the enzyme.

The peroxidase was prepared according to the method of Willstätter and Stoll [1918]. Horseradish was used as the source of the enzyme, owing to the absence of aerobic oxidases other than peroxidase.

2.5 kg. of horseradish was cut into slices about 1 mm. thick, and dialysed in running tap-water for 6 days. The peroxidase was then precipitated on the

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plant tissue by treatment with oxalic acid, and the slices were minced to a porridge-like mass from which the enzyme was liberated with baryta. The active extract of $1\frac{1}{4}$ litres was freed from barium by passing carbon dioxide into the solution, and a large amount of protein was precipitated by the addition of 0.9 vol. of alcohol. The clear filtrate was evaporated under reduced pressure to a volume of 30 cc. and, after removal of barium carbonate by filtration, the peroxidase was precipitated in the form of yellow flakes by the addition of 5 vols. of alcohol. The precipitate was centrifuged, washed with alcohol and dried. The product was a yellow powder 0.9 g. in weight. In this form the enzyme was quite stable, but in aqueous solution it lost 5–10 % of its activity in 24 hours at room temperature.

The activity of the preparation.

The activity of the preparation was estimated by determining the purpurogallin number, which is defined by Willstätter and Pollinger [1923] as the mg. of purpurogallin which 1 mg. of enzyme preparation will form in 5 minutes from 5 mg. of pyrogallol and 50 mg. of hydrogen peroxide dissolved in 2 litres of water at 20°, when the amount of enzyme is so small that the change in substrate concentration during the reaction time is practically negligible.

The reaction was stopped at the end of 5 minutes by the addition of dilute sulphuric acid, and the purpurogallin was extracted with ether, the ethereal extracts being made up to a total volume of 500 cc. The amount of purpurogallin present was then estimated in a colorimeter, the standard for comparison consisting of a solution of purpurogallin containing 100 mg. of purpurogallin in a litre of ether. The purpurogallin for the standard was prepared by the method of Perkin and Steven [1903], and was recrystallised from alcohol and ether.

As the result of a large number of estimations an average purpurogallin number of 70 was obtained, compared with an average value of 145 obtained by Willstätter and Pollinger for preparations at the same stage in the purification.

The temperature coefficient.

As previously stated, Haldane [1930] suggested that at sufficiently low concentrations of hydrogen peroxide peroxidase would have a temperature coefficient of unity. The hypothesis depends on the fact that peroxidase is capable of activating many times its weight of hydrogen peroxide per second. Thus Willstätter and Pollinger's best peroxidase preparation could activate 1000 times its weight of hydrogen peroxide per second at 20° when used on leucomalachite green, which is oxidised 120 times as fast as pyrogallol. The hydrogen peroxide concentration was 2.5 mg. per litre (Willstätter and Weber, 1926, 1].

It is extremely desirable when comparing the reaction velocities of peroxidase at different temperatures to take the necessary readings while the

reaction is following a linear course. Hence the change in substrate concentration during the reaction time must be negligible. Now with the purpurogallin method concentrations of the dye of less than 1 mg./100 cc. cannot be measured, and when the hydrogen peroxide concentration is extremely small all the latter may be used up before the necessary amount of purpurogallin is obtained. Thus the method is unsuitable for measurement of the temperature coefficient of peroxidase at low concentrations of hydrogen peroxide. The colour intensity of the triphenylmethane dyes is much greater than that of purpurogallin, and a method using leucomalachite green as acceptor has been found very reliable by Willstätter and Weber [1926, 1]. The procedure is much simpler than that of the purpurogallin method, as it is unnecessary to extract the dye with ether, the amount of malachite green which is formed by the action of the enzyme on the leuco-base being estimated colorimetrically in the reaction mixture itself. The chief disadvantage of the method is the insolubility of the leuco-base, which makes it impossible to work at a reaction much less acid than p_H 4.

The leucomalachite green was prepared by the condensation of benzaldehyde and dimethylaniline in presence of anhydrous zinc chloride, and was repeatedly recrystallised from alcohol and ether, the final product being quite white. A stock solution of reducing substrate was prepared by saturating $N/20$ acetic acid with the leuco-base *in vacuo*. The resulting solution contained approximately 10 mg. of leucomalachite green per 100 cc., and remained colourless for several weeks if kept *in vacuo*. The reaction mixture was made up of 10 cc. of this solution, 0.2 cc. of 0.166 N sodium acetate which adjusted the reaction to p_H 4, the required amount of hydrogen peroxide, 1 or 2 cc. of the enzyme solution prepared by dissolving a few mg. of the powder in 100 cc. of water, and sufficient distilled water to make the volume up to 15 cc. The reaction was carried out in a water-bath at the required temperature for a time varying between 2 and 8 minutes, at the conclusion of which the enzyme was destroyed by the addition of 1 cc. of N sulphuric acid, which after 30 seconds was neutralised with an equivalent amount of sodium carbonate solution. The mixture was shaken to remove carbon dioxide, and the malachite green produced by the reaction estimated in a colorimeter against a standard solution prepared by dissolving 10 mg. of malachite green in a litre of $N/20$ acetic acid. The acetic acid inhibits the hydrolysis of the dye and thus prevents fading of the standard solution.

The range of hydrogen peroxide concentrations which could be used while allowing the experimental readings to be made during the linear course of the reaction was first determined. Fig. 1 illustrates the course of the reaction in presence of different concentrations of hydrogen peroxide at p_H 4. The amount of malachite green formed is plotted against the time. In this case the curves were obtained by placing the reaction solution in the colorimeter cup immediately after the addition of the enzyme, and observing the depth of colour at various times, and thus a complete curve could be drawn for one

solution. The reaction is comparatively slow at a concentration of hydrogen peroxide of $6 \times 10^{-3} M$, reaches a maximum at $6 \times 10^{-5} M$, and there is little change in velocity between concentrations of $6 \times 10^{-4} M$ and $6 \times 10^{-6} M$, after which the velocity falls off rapidly as the hydrogen peroxide concentration is further decreased. The curves are all linear except in the case of

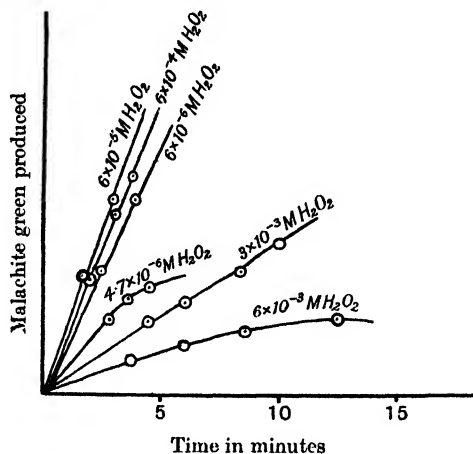


Fig. 1.

the highest concentration where the slight falling off in velocity is probably due to enzyme destruction caused by the high concentration of hydrogen peroxide, and in the lowest concentration where the falling off is due to the using up of a large proportion of the hydrogen peroxide during the course of the reaction.

Having thus found the range of hydrogen peroxide concentrations which it was practicable to use, a number of reaction solutions were made up containing different amounts of peroxide, placed in a water-bath at 20° , and after a definite time the malachite green produced was estimated according to the method already described. The p_s curve was obtained by plotting the reaction velocity against the logarithm of the substrate concentration. The result is shown in Fig. 2. It will be observed that the curve is approximately symmetrical. A possible explanation of its form is suggested in the second part of this work. An advantage of plotting the substrate concentration logarithmically is that the points corresponding to low concentrations which are important in determining the Michaelis constant K_m , that is, the substrate concentration at which half the maximum velocity is reached, are not crowded together. From the curve the value of the apparent affinity of the enzyme for hydrogen peroxide may be obtained, the Michaelis constant, which is the reciprocal of the affinity, being $5 \times 10^{-6} M$, which is in agreement with the value of $6 \times 10^{-6} M$, calculated by Haldane from the data of Willstätter and Weber [1926, 1].

Willstätter and Weber showed that the Q_{10} of peroxidase at a concentration of peroxide of 25 mg. per litre ($7.4 \times 10^{-4} M$) was approximately 2. In order to test the hypothesis of Haldane it was decided to see if this was still the case at a concentration of approximately $10^{-6} M$. At this concentration it was found impossible to obtain readings while the reaction was following

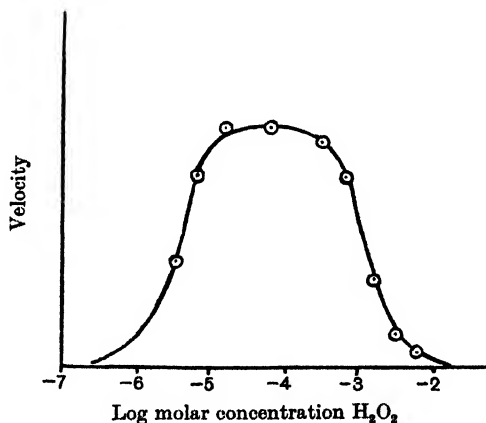


Fig. 2.

a linear course, using the original method of estimation, and to make this possible the method of estimation was altered. 100 cc. of the leucomalachite green solution were used instead of 10 cc., and the malachite green was extracted twice with amyl alcohol, the combined extracts being made up to a total volume of 10 or 15 cc. By this means the dye intensity could be increased 10-fold. The standard solution for comparison in the colorimeter was obtained by extracting the original standard with an equal volume of amyl alcohol.

By means of this method curves were obtained at a concentration of hydrogen peroxide of $1.3 \times 10^{-6} M$ showing the relative velocities of the

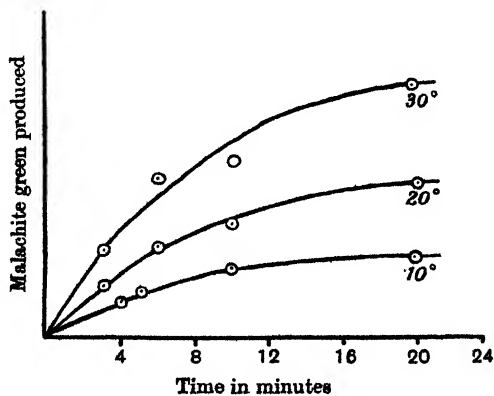


Fig. 3.

reaction at temperatures of 10°, 20° and 30°. It will be observed (Fig. 3) that the reaction follows a linear course for a considerable period, and thus it is possible to obtain the initial velocities at the three temperatures without drawing the tangents to the curves. The curves give Q_{10} 1.9 between 10° and 20°, and 1.8 between 20° and 30°, and thus, contrary to the suggestion of Haldane, the temperature coefficient appears to have been little affected by the change in hydrogen peroxide concentration.

The question was further studied by comparing the p_s curves obtained at different temperatures. If the temperature coefficient is different for different hydrogen peroxide concentrations, one would expect an alteration in the shape of the p_s curve on the horizontal as well as the vertical scale, but, on the other hand, if the temperature coefficient is independent of hydrogen peroxide concentration, the only effect of increase of temperature should be to increase the vertical without altering the horizontal scale. p_s curves were therefore obtained at temperatures of 0°, 20° and 30°, and are shown in Fig. 4. The

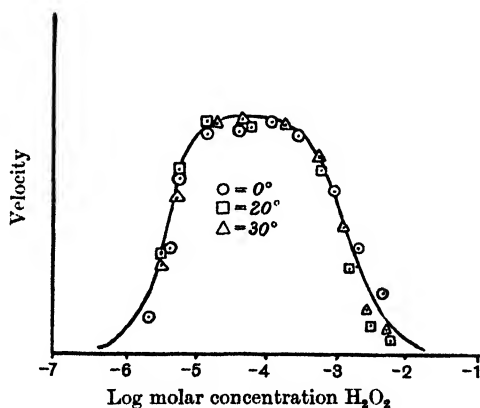


Fig. 4.

curves have all been reduced to the same vertical scale the better to illustrate the small change in affinity. It is obvious that there is little change in the horizontal range of the curves and thus one would expect the temperature coefficient to remain at almost the same figure even at still lower peroxide concentrations than those employed.

Dann [1931], working with citric acid dehydrogenase, has successfully used the temperature coefficient of the affinity as a means of estimating the heat of formation of the enzyme substrate compound. From the curves of Fig. 4 it is obvious that the temperature coefficient of the affinity of peroxidase for hydrogen peroxide is too small to be accurately measured, and thus the heat of formation of the enzyme-substrate compound must be very small.

Haldane's prediction was based upon the assumption that the gas laws could be applied to a substance dissolved in a liquid, so that the frequency of collisions could be approximately deduced by regarding the substance as

in the gaseous state under a pressure equal to its osmotic pressure in solution. The results of this work do not bear out the assumption and may be regarded as evidence that the frequency of collisions in the liquid state is at least several hundred times as great as that indicated by an application of the ordinary gas laws.

The object of the second part of this work was to investigate the relations between the hydrogen peroxide and the reducing substrates at the enzyme. Many years ago Bach [1904], working on the oxidation of hydriodic acid by peroxidase, showed that excess of hydrogen peroxide inhibited the reaction. He attributed the inhibition to irreversible destruction of the enzyme. Willstätter and Weber [1926, 2] investigated this inhibition, and showed that while irreversible destruction of the enzyme may be caused by high concentrations of hydrogen peroxide, reversible inactivation is also produced. In such cases the velocity may be increased either by increasing the concentration of reducing substrate (pyrogallol or leucomalachite green), or by removing some of the hydrogen peroxide by means of catalase. They suggest as an explanation of the reversible inhibition that the peroxide is able to combine with the enzyme in either of two ways, giving an active and an inactive enzyme-substrate compound respectively. Thus in presence of low concentrations of hydrogen peroxide the peroxide combines with the enzyme

in the form $\begin{array}{c} \text{H} \\ \diagup \\ \text{H} \end{array} \text{O} = \text{O}$ to give an unstable compound which is catalytically active. In high concentrations, however, it combines as $\text{H}-\text{O}-\text{O}-\text{H}$, giving a relatively stable enzyme-substrate compound which is catalytically inactive.

This explanation is unsatisfactory, since by the law of mass action the ratio of the two forms of the enzyme-substrate compound should be independent of the substrate concentration. On the basis of the results obtained by the author an alternative hypothesis is put forward. This was suggested by the addition compound theory of enzyme action [Woolf, 1931], according to which for an enzyme reaction to occur all the substrates must be combined at the enzyme. The explanation depends on the assumption that the reducing substrate combines with the enzyme, a view which has hitherto not been accepted. Haldane [1930] summing up the evidence on this question writes "The reducing substrates of peroxidase appear to act, not by combining with it as a preliminary to oxidation, but by combating the inhibition of its activity by the relatively high hydrogen peroxide concentrations used." The results obtained, however, indicate that the reducing substrate does combine with the enzyme. It is further postulated that the hydrogen peroxide combines with the enzyme at a group which is moderately specific, as it can only be replaced by compounds of related structure such as ethyl hydrogen peroxide [Wieland and Sutter, 1930]. The group with which the reducing substrate combines has a far wider range of specificity, as shown by the variety of substances which can be oxidised by the peroxidase system. It is suggested that hydrogen peroxide is also capable of combining at the latter group. When

both peroxide and reducing substrate are combined at the enzyme at their respective groups, an active enzyme-substrate compound is produced, but in presence of excess of hydrogen peroxide the latter may combine with the enzyme at the non-specific group, thus keeping the reducing substrate from the enzyme and causing inhibition. Such inhibition would be reversible and could be combated by either of the methods described by Willstätter and Weber, *i.e.* by raising the concentration of reducing substrate or by removing some of the hydrogen peroxide with catalase. In both these cases the reducing substrate would be in a better position to compete with the peroxide for the enzyme and thus the reaction velocity would be increased.

The relations between the hydrogen peroxide and the reducing substrate at the enzyme were first studied by obtaining a series of p_s curves each at a different concentration of leuco-malachite green. Now at p_H 4 a saturated solution of leucomalachite green contains only 10 mg. of the base in 100 cc., and thus it is impossible to obtain the wide range of concentrations of reducing substrate which is desirable. At more acid reactions, however, the solubility is considerably increased, being about 10 times as great at p_H 3.2, at which reaction the work to be described was carried out. The leucomalachite green was dissolved in *N* acetic acid, and the reaction was adjusted to p_H 3.2 with sodium acetate.

Fig. 5 shows the series of peroxide p_s curves obtained in presence of

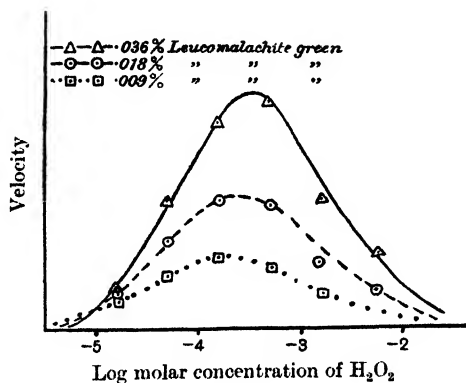


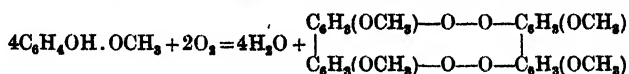
Fig. 5.

different concentrations of leucomalachite green. In the case of a simple enzymic reaction, the curve obtained by plotting the substrate concentration is a rectangular hyperbola or Michaelis curve, or if the substrate concentration is plotted logarithmically an S-shaped curve results which is of the same form as when the amount of dissociation of a weak acid is plotted against p_H . In the case of peroxidase a similar curve would be expected if there were no competition between the two substrates. It will be observed that the p_s curves obtained with peroxidase, shown in Fig. 5, do not resemble the typical form of p_s curve just described. They are symmetrical curves similar to those

obtained when the dissociation residues of ampholytes are plotted against p_H . As the peroxide concentration is increased the velocity rises rapidly until an optimum is reached, after which further increase in concentration causes a rapid fall in reaction velocity. It is suggested that this fall in velocity is due to the competitive combination of hydrogen peroxide at the non-specific grouping of the enzyme. This view is further supported by consideration of the effect of increasing the concentration of reducing substrate. As the concentration of reducing substrate is increased the range of peroxide concentration over which activity is obtained is extended, the system being catalytically active in higher concentrations of peroxide. The curves obtained, however, are still approximately symmetrical, as the position of the optimum is correspondingly shifted. Besides the extension of the horizontal range of the curves, the vertical range is also extended by increase in the leucomalachite green concentration. The increase in reducing substrate concentration has a marked activating effect on the velocity when the peroxide is present in such concentration as formerly produced inhibition. These facts are in complete accordance with the hypothesis outlined above.

Leucomalachite green was used as the reducing substrate in the work on the temperature coefficient because, owing to its colour intensity, the dye produced could be estimated in very low concentrations. For the present work, however, its insolubility rendered it unsuitable. It was therefore decided that further work on the subject should be done with another more soluble substrate. Nitrite, which is oxidised by the peroxidase system to nitrate, and quinol, which is oxidised to quinone, were both tried, but had to be discarded owing to the difficulties of accurately estimating, in presence of hydrogen peroxide, the small amounts of nitrate and quinone produced.

The reducing substrate finally chosen was guaiacol, which, according to Bertrand [1903], is oxidised to a complex red compound, tetraguaiacol.



The method of estimation employed was that of Bansi and Ueko [1926]. The method is a colorimetric one, essentially similar to the leucomalachite green method, but the standard solution for comparison in the colorimeter is an artificial one, made by mixing cobalt nitrate solution and potassium dichromate solution until the correct tint is obtained. The reaction is stopped by the addition not of acid, which causes the colour to fade rapidly, but of mercuric chloride solution.

Employing this method, the series of peroxide p_s curves shown in Fig. 6 was obtained at p_H 4.7, using four different concentrations of guaiacol. The curves are similar to those obtained with leucomalachite green (Fig. 5), being symmetrical and resembling in form the dissociation residue curves of ampholytes. Increase in guaiacol concentration has the same effect as increase in leucomalachite green concentration, the curves being extended in range both

horizontally and vertically. The increase of guaiacol concentration shows an activating effect on the velocity when the peroxide is present in concentration so high as formerly to have produced an inhibition. This was also the case with leucomalachite green. These facts afford additional support for the

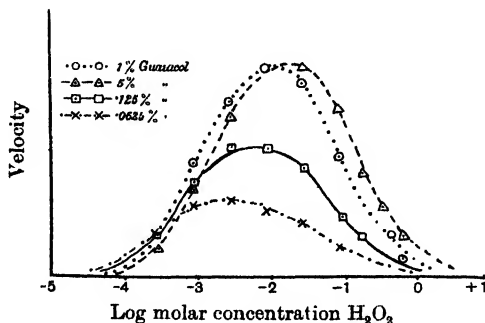


Fig. 6.

theory that the peroxide and reducing substrate compete for the relatively non-specific group of the enzyme. It will be observed that with the highest concentration of guaiacol the velocity at low concentrations of peroxide is less than that when the concentration of guaiacol is 0.5 %, though in higher concentrations of peroxide an activation is produced. This was not observed with leuco-malachite green, possibly because, owing to the insolubility of the latter compound, the necessary concentration to produce the effect could not be obtained.

This phenomenon was further studied in a different way. A series of curves was obtained at p_H 4.7 showing the relation between the concentration of guaiacol and the initial reaction velocity, in presence of several constant concentrations of peroxide. These are shown in Fig. 7. A true Michaelis curve

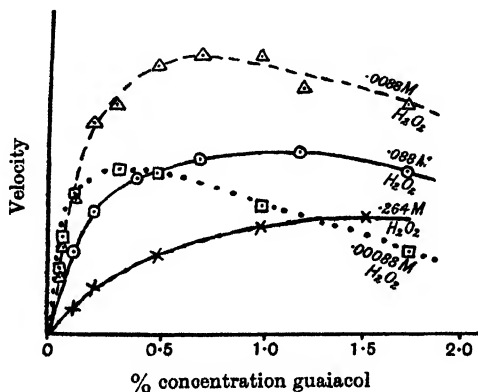


Fig. 7.

is not obtained. At the highest peroxide concentration employed, 0.264 M , the optimum concentration of guaiacol has not been reached, the reaction

velocity still increasing with increase in guaiacol concentration. At a concentration of $0.088 M$ peroxide, an optimum guaiacol concentration can be reached, but as the guaiacol is further increased, the reaction velocity, instead of remaining constant, begins to decrease. This fall in velocity is better seen in the other two curves, in presence of still lower peroxide concentrations. As the concentration of peroxide is decreased the optimum velocity is obtained at progressively lower guaiacol concentrations. These curves are shown plotted logarithmically in Fig. 8. They are approximately symmetrical, and again resemble dissociation residue curves of ampholytes.

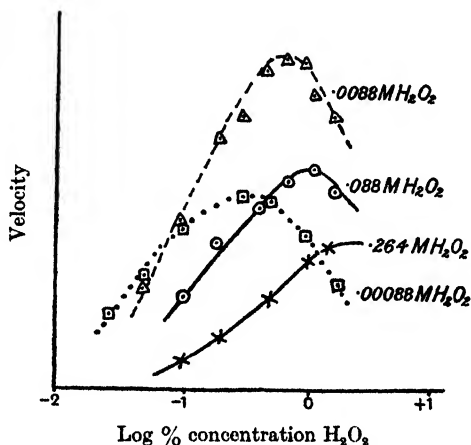


Fig. 8.

The curves of Figs. 7 and 8 cannot be completely explained by the simple theory that the peroxide competes with the reducing substrate at the enzyme, and some extension of the theory is accordingly necessitated. The form of the curves does not seem to support the possibility that the reducing substrate competes with the peroxide at what has been assumed to be the specific combining group of the enzyme. A more adequate explanation which fits the curves, at any rate qualitatively, is to be found from a study of the following considerations.

Simple enzyme p_s curves are S-shaped, *e.g.* that of saccharase, further increase in substrate concentration after the maximum velocity has been reached having little effect on the velocity until, in the case of saccharase, the substrate concentration is so high that water becomes a limiting factor [Nelson and Schubert, 1928]. In the case of other enzymes however, and in particular of lipase, after the optimum velocity is reached, further increase in substrate concentration causes a rapid fall in the velocity of the reaction, approximately symmetrical curves being obtained, as was observed, *e.g.* by Bamann [1929], for the velocity of hydrolysis of ethyl *d*-mandelate by dog-liver lipase. Haldane [1930] has suggested that such a phenomenon may be explained by the assumption that the substrate is capable of combining

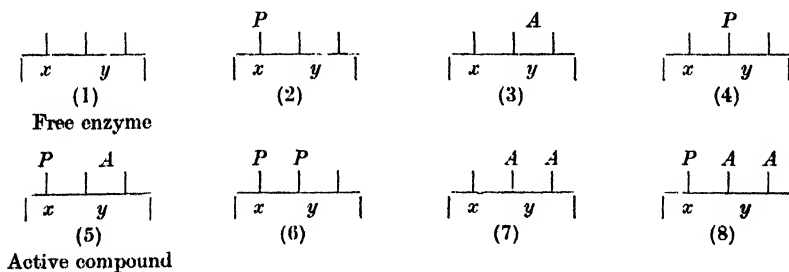
twice with the enzyme, giving an inactive compound. Whereas in the case of the normal enzyme-substrate compound ES , the enzyme is united to the ester by two linkages, one at the alcohol, the other at the acid residue, in the case of the ES_2 compound each substrate molecule is only once united, and hence the strain producing hydrolysis does not arise. This is illustrated by the diagrams shown below which are taken from a paper on lipase by Murray [1930].



Murray tested the suggestion of Haldane using the esterase of sheep-liver, and found that it provided a satisfactory explanation of his experimental results.

Such a hypothesis affords an adequate explanation of the action of excess of reducing substrate in producing inhibition of peroxidase action. It is therefore suggested that each molecule of reducing substrate normally combines at two linkages with the enzyme, forming a compound which, when combined with peroxide at the requisite group, gives a catalytically active enzyme-substrate compound. The reducing substrate is, however, also capable of combining in a different way with the enzyme, forming an inactive compound ES_2 , a molecule combining with each of the two linkages across which one molecule normally combines. In presence of excess of reducing substrate this inactive compound is naturally produced to a large extent and thus inhibition of the reaction is caused.

On this hypothesis, in a solution of peroxidase, peroxide, and reducing substrate at least 8 forms of the enzyme may be present. These are shown in the scheme below, in which the combining groups of the enzyme are denoted by x (the group specific for peroxide), and y , the peroxide by P , and the reducing substrate or acceptor by A .



The active enzyme-substrate compound is denoted by 5. In presence of excess of hydrogen peroxide, a large amount of the inactive compounds 4 and 6 will be produced, and thus inhibition will be caused. In presence of excess of reducing substrate a large proportion of the enzyme will be combined to form the compounds 7 and 8, which are also inactive and produce inhibition. This appears to be the simplest explanation of the results obtained.

SUMMARY.

The suggestion of Haldane [1930] that the temperature coefficient of peroxidase action would be approximately unity at very low concentrations of hydrogen peroxide has been tested and found to be erroneous. The temperature coefficient is just under 2 at all hydrogen peroxide concentrations.

The effect has been studied of variation of the concentrations of hydrogen peroxide and the reducing substrate on the velocity of peroxidase action, the reducing substrates used being leucomalachite green and guaiacol. The results suggest that both peroxide and reducing substrate must be combined at the enzyme before catalysis can take place.

I wish to thank Prof. J. B. S. Haldane and Dr B. Woolf for much help and advice, and Sir F. G. Hopkins for his interest and encouragement during the course of this work. My thanks are also due to the Department of Scientific and Industrial Research for a part-time grant.

REFERENCES.

- Bach (1904). *Ber. deutsch. chem. Ges.* **37**, 3787.
Bamann (1929). *Ber. deutsch. chem. Ges.* **62**, 1538.
Bansi and Ucko (1926). *Z. physiol. Chem.* **157**, 192.
Bertrand (1903). *Compt. Rend. Acad. Sci.* **134**, 124.
Dann (1931). *Biochem. J.* **25**, 177.
Haldane (1930). *Enzymes* (Longmans, London).
Murray (1930). *Biochem. J.* **24**, 1890.
Nelson and Schubert (1928). *J. Amer. Chem. Soc.* **50**, 2188.
Perkin and Steven (1903). *J. Chem. Soc.* **83**, 192.
Wieland and Sutter (1930). *Ber. deutsch. chem. Ges.* **63**, 67.
Willstätter and Stoll (1918). *Liebig's Ann.* **416**, 21.
—— and Pollinger (1923). *Liebig's Ann.* **430**, 269.
—— and Weber (1926, 1). *Liebig's Ann.* **449**, 156.
—— (1926, 2). *Liebig's Ann.* **449**, 175.
Woolf (1931). *Biochem. J.* **25**, 342.

CIV. INVESTIGATIONS ON THE PREPARATION AND BEHAVIOUR OF VITAMIN B₁ CONCENTRATES FROM YEAST.

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INTRODUCTION.

IN a previous publication a method was described for the concentration of vitamin B₁ from wheat embryo, which had been attended with a considerable measure of success [Guha and Drummond, 1929]. A semi-crystalline preparation was obtained, of which the pigeon-curative day-dose was found to be of the order of 0.005 mg. It also promoted good growth in young rats, placed on a vitamin B₁-deficient diet, in a daily dose of 0.015 mg. The preparation, however, was obviously impure, and when subjected to fractionation by gold chloride behaved as if the vitamin had been split into two fractions, as a mixture of the substances obtained from the gold precipitate and filtrate appeared to be more active than either. As, however, a very small amount of material was at our disposal, the number of pigeon and rat tests was very inadequate, and, therefore, our results had to be taken with reserve. Recently, however, Reader [1930] has stated that the experimental "beriberi" condition in rats is a complex syndrome, resulting from a deficiency of two factors, B₁ and B₄. Furthermore, the crystals obtained by Jansen and Donath [1926] from rice polishings, which were originally claimed to be vitamin B₁ hydrochloride, have also been stated to contain vitamin B₄ [Jansen, Kinnersley, Peters and Reader, 1930]. Further information on this subject will be awaited with interest.

The present investigation was undertaken with the double object of finding out how far the method of fractionation, which had been previously applied to wheat embryo, could be successfully employed for the concentration of the vitamin from yeast, and whether any evidence about the multiple nature of vitamin B₁ could be obtained when yeast was used in place of wheat embryo as the raw material. Brewery bottom yeast was employed by Seidell [1926] for the concentration of vitamin B₁, but as his method of biological assay depended on the prevention of loss of weight of pigeons on a polished rice diet, it is clear from recent work on pigeons that the tests were complicated by other factors besides B₁ [Williams and Waterman, 1928; Carter, Kinnersley

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and Peters, 1930, 1, 2; Guha, 1929]. Baker's yeast has usually been employed by Peters and his co-workers and he has recently published improvements in his technique by fractional precipitation by means of phosphotungstic acid at different hydrogen ion concentrations [Kinnorsley and Peters, 1930].

The fractionation recorded in the present paper was carried out with brewer's top yeast, as its aqueous extract was found by means of preliminary experiments, based on rat-growth tests, to be somewhat richer in vitamin B_1 than an aqueous extract of baker's yeast. The method has been standardised up to the platinum stage by several repetitions. The preparation obtained at this stage promotes good growth in young rats in a daily dose of 0.075–0.10 mg. It is expected that the material collected by this method of fractionation in several operations might be further fractionated with advantage. Incidentally the relation, if any, between vitamin B_1 on the one hand and secretin, co-enzyme and bios on the other has been investigated. The absorption spectra and certain other properties of B_1 concentrates are also described.

EXPERIMENTAL.

Biological technique.

The method of biological assay described before [Guha and Drummond, 1929] has been used in these experiments with a few modifications. Young rats, about 50 g. in weight, were fed on a basal diet of the following composition:

Rice starch	65 %
"Light white casein" (B.D.H.)	21 %
Palm kernel oil	10 %
Salt mixture (Osborne and Mendel)	4 %

This diet was supplemented daily with 2 drops of cod-liver oil and 1.5 cc. of a 50 % solution of autoclaved marmite in order to supply vitamins A. D and B_2 . The latter preparation was obtained by mixing the commercial yeast extract, marmite, with baryta, bringing the mixture roughly to p_H 9 and autoclaving for $1\frac{1}{2}$ hours at 124° , barium being finally removed by means of sulphuric acid. Numerous experiments have proved that such a preparation contains large quantities of vitamin B_2 and negligible quantities of B_1 . When the weight of the young animals began to decline on this diet they were fed with the vitamin B_1 fraction to be tested, and the standard of growth was taken to be at least 10–12 g. per week. Each fraction was tested on at least 2 rats, usually more. Rat-growth tests were adopted as a matter of routine, while these were periodically checked by pigeon-curative tests. The pigeon-curative day-dose was computed by dividing the amount injected by the number of days for which protection was afforded.

The technique of assaying vitamin B_1 has recently been discussed by several investigators [Smith, 1930; Kinnorsley, Peters and Reader, 1930].

Smith describes an apparently promising method, based on curative tests with rats, by which he seems to have obtained very regular results. In our experience, rat-growth tests have yielded fairly uniform results. Fig. 1 illustrates the fact that under strictly controlled conditions it is possible to get

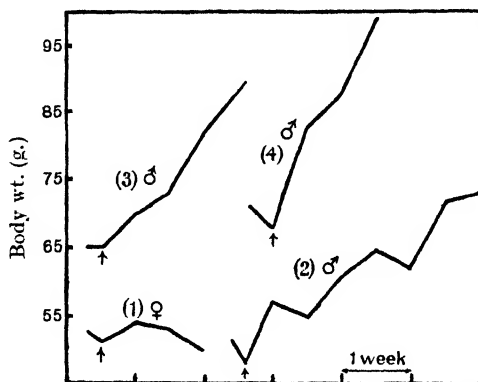


Fig. 1. Experiments with an aqueous-alcoholic extract of brewer's yeast.

Curve (1). ↑ dose = 0.15 cc.

Curve (2). ↑ dose = 0.225 cc.

„ (3). ↑ dose = 0.33 cc.

„ (4). ↑ dose = 0.45 cc.

approximately proportionate growth responses to graded doses of a given vitamin B₁ preparation. We have adhered to this method in order to have results comparable with those obtained from wheat germ. It is possible, however, that at the last stages of fractionation, Smith's curative method might give more trustworthy results. It would, doubtless, be more economical.

Tests with known substances.

In the earlier paper, it was stated that various substances like nucleic acid, betaine, nicotinic acid, which had at one time or other been credited with properties of B₁, were found to be inactive by the rat-growth tests. The following substances have now been fed in the doses stated, again with negative results.

Substance tested						Dose (mg.)	
Adenine picrate	2	and 4
Guanine hydrochloride	2	„ 4
Uracil	2	„ 4
Synthetic <i>dl</i> -thyroxine	0.5	„ 1
Histamine acid phosphate	0.1	„ 0.2
“Adrenalinum” (B.D.H.) (faintly acidified with HCl)	0.1	„ 0.2

The relative vitamin B₁ values of extracts of brewer's top yeast and baker's yeast.

Aqueous extracts of brewer's top yeast and baker's yeast, made by extraction with boiling water and boiling for 3 minutes were, compared for vitamin B₁ activity. The brewer's yeast gave somewhat more active extracts

in most cases. One sample of baker's yeast was, however, found particularly rich in vitamin B₁ (Fig. 2). It is probable that there is a considerable variation

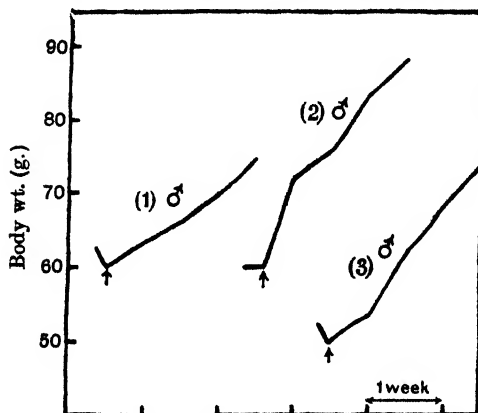


Fig. 2. Tests with aqueous extracts of brewer's top yeast and baker's yeast.
 Curve (1). ↑ dose \equiv 0.5 g. baker's yeast.
 „ (2). ↑ dose \equiv 0.5 g. brewer's top yeast.
 „ (3) illustrates the potency of a particularly active variety of baker's yeast;
 ↑ dose \equiv 0.5 g. yeast.

in the vitamin B₁ potency of different samples of both varieties of yeast [see also Quinn, Whalen and Hartley, 1930]. Pirie's method of extraction of yeast for glutathione [Pirie, 1930] also extracts vitamin B₁ effectively from both brewer's and baker's yeast.

Procedure of fractionation.

Brewer's yeast was employed for the fractionation of vitamin B₁ recorded in this paper. This method has met with success up to the platinum stage every time it was tried, though, unfortunately, the same degree of concentration was not achieved at this stage as had been obtained with wheat germ [Guha and Drummond, 1929]. The following account represents a fairly standardised method.

Stage 1. Extraction with aqueous alcohol. 3178 g. of fresh brewer's top yeast were extracted with 2.5 l. of 95 % alcohol at 60–70° for 4–6 hours. The residue, after filtration, was re-extracted with 1.75 l. of 50 % alcohol at the same temperature for the same period. After concentration under reduced pressure, the first extract was active in a daily dose, representing 0.64 g. of the fresh undried yeast, which contained 40 mg. total solids, of which 33.5 mg. were organic. The active dose of the second extract was equivalent to 1.6 g. of the fresh yeast and carried 25 mg. of the total solids, of which 22.4 mg. were organic (Fig. 3).

Stage 2. Fractionation by lead acetate. The two extracts of the preceding stage were combined and precipitated with 120–150 g. of neutral lead acetate in aqueous solution. The filtrate from the lead precipitate was freed from lead

by hydrogen sulphide in a slightly acid medium, the hydrogen sulphide being later removed by concentration *in vacuo* to a volume of 1500 cc. This material was active in daily doses containing 40 mg. of total solids, of which 30 mg. were organic, equivalent to 0.75 g. of the fresh yeast (Fig. 3).

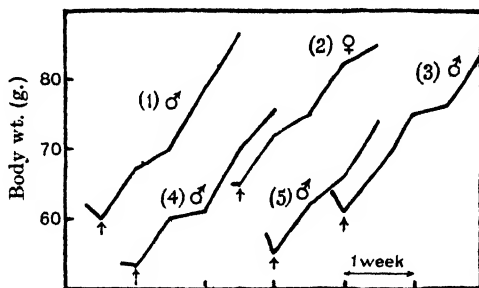


Fig. 3.

- Curve (1). The 95 % alcoholic extract of brewer's yeast; ↑ dose \equiv 0.64 g. of fresh yeast.
 „ (2). The second 50 % alcoholic extract of brewer's yeast; ↑ dose \equiv 1.6 g. of fresh yeast.
 „ (3). The lead filtrate; ↑ dose \equiv 0.75 g. yeast.
 „ (4). The fuller's earth extract; ↑ dose \equiv 1.38 g. yeast.
 „ (5). Silver nitrate-baryta fraction at p_H 6.8; ↑ dose \equiv 1.5 g. yeast.

Pigeon-curative tests with 4 birds showed that the average day-dose contained 12 mg. total solids of which 9.3 mg. were organic.

Stage 3. Adsorption by fuller's earth. The lead filtrate was brought, if necessary, to p_H 4.5 and agitated for 20 minutes with 50 g. fuller's earth¹ and left overnight. The supernatant fluid was syphoned off and the bottom layer sucked dry. The fuller's earth residue, which weighed 67 g., was extracted by grinding up with 200 cc. of saturated baryta and immediately filtering with strong suction. The total operation of grinding and filtering took 5–7 minutes. The filtrate was received in a slight excess of concentrated sulphuric acid. The fuller's earth residue was washed with a little baryta and then with water. After the removal of the precipitated barium sulphate from the filtrate, the fraction proved to be active in doses containing 1.4 mg. total solids, of which 1.3 mg. were organic. The dose was equivalent to 1.38 g. yeast (Fig. 3). Curative tests with 3 pigeons showed that the average day-dose contained 0.57 mg. total solids of which 0.53 mg. was organic.

Stage 4. Fractionation by silver nitrate and baryta. The above fuller's earth extract, which was acid to Congo red, was treated with 15 cc. of 50 % silver nitrate solution, which represented an excess, and then treated with the requisite quantity of saturated baryta to bring the p_H to 4.5 and allowed to settle for 2 hours. The precipitate which was very slightly active was discarded

¹ In our experience different samples of fuller's earth have been found to differ greatly in their adsorbing capacity. It is therefore desirable to stock a quantity of fuller's earth, after its suitability has been established. In these experiments Boots's fuller's earth was used. Experiments with the "acid clay" used by Jansen and Donath show that it is also a very efficient adsorbent for the vitamin.

and the filtrate brought to p_H 6.8 by adding the requisite amount of saturated baryta. After being allowed to stand overnight, the brown precipitate was filtered off (wet weight 5 g.) and decomposed by hydrochloric acid. On filtration, a straw-coloured filtrate was obtained, of which the rat day-dose contained 0.345 mg. total solids, of which 0.29 mg. was organic matter (Fig. 3). This was equivalent to 1.5 g. yeast. Further fractionation of the main filtrate at p_H 8 yielded negative results. Curative tests on 2 pigeons with the silver fraction precipitated at p_H 6.8 showed that the average day-dose contained 0.16 mg. total solids of which 0.13 mg. was organic.

Stage 5. Fractionation by phosphotungstic acid. The fraction obtained at p_H 6.8, which is thus seen to contain almost all the activity, had a volume of 200 cc., and this was treated with the required quantity of concentrated H_2SO_4 to make roughly 5 % acid, and then with 14 cc. of a saturated solution of phosphotungstic acid in 5 % H_2SO_4 , which represented a slight excess. The precipitate was removed after 24 hours, filtered under strong suction (dry weight = 3.5 g.), dissolved in 70 cc. of 50 % acetone and filtered from a very small amount of undissolved residue. The clear red-brown filtrate was treated gradually with 50 cc. of 5 % H_2SO_4 with constant stirring. At this stage a slight turbidity remained, which did not disappear on warming to 60°. A gummy material separated, which, on leaving the mixture for a week in the refrigerator, turned granular. This orange-yellow precipitate (a) was filtered, sucked dry (weight = 1 g.) and decomposed by dissolving in 50 cc. of 50 % acetone and shaking up with 30–40 cc. baryta, which were sufficient to make the solution alkaline to phenolphthalein. The precipitate of barium phosphotungstate was filtered off immediately and the filtrate acidified with sulphuric acid. After filtration from $BaSO_4$ and the removal of acetone *in vacuo* the clear pale straw-coloured filtrate proved to be active in a daily dose containing 0.29 mg. total solids, equivalent to 1.6 g. yeast (Fig. 4)¹. The filtrate obtained after removal of precipitate (a) on treatment with an excess (300 cc.) of 5 % H_2SO_4 yielded a further quantity of precipitate, which was found to be inactive.

The filtrate obtained after the removal of the original phosphotungstic precipitate, on standing in the refrigerator for several days, deposited a very small amount of colourless crystals which appeared as a homogeneous mass of rhombohedra under the microscope. After decomposition by baryta, it proved to be entirely inactive. On evaporation and examination it was found to be crystalline and homogeneous. In view of the small amount of material, however, its identity was not further investigated.

¹ It is not easy to understand why Kinnersley and Peters [1930] failed to obtain the vitamin in the acetone-soluble phosphotungstic fraction. The phosphotungstates of the vitamin fractions from wheat germ [Guha and Drummond, 1929], from rice polishings [Jansen and Donath, 1926], as also from brewer's yeast (as shown in the present communication) are apparently acetone-soluble. It is possible, however, that this solubility is not a property of the vitamin *per se* but is due to the presence of the acetone-soluble phosphotungstates of other materials, which may not be precipitated under the somewhat altered conditions of Kinnersley and Peters.

The average pigeon-curative day-dose of the active phosphotungstic acid fraction, tested with 3 birds, contained 0.052 mg. total solids.

Stage 6. Extraction with absolute alcohol. The above active phosphotungstic acid fraction was evaporated to dryness in a vacuum desiccator containing soda lime and was extracted four times with hot (70–80°) absolute alcohol. The pale straw-coloured extract, after filtration, measured 116 cc., and a portion of it, after removal of alcohol, was found to be active in doses containing 0.274 mg. total solids, equivalent to 1.76 g. yeast (Fig. 4).

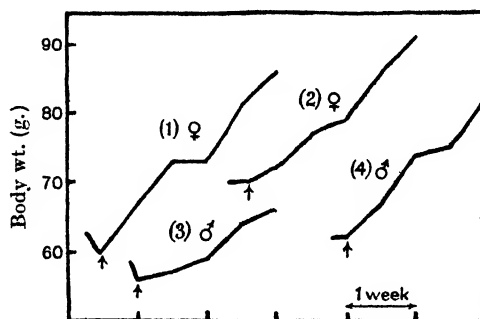


Fig. 4.

- Curve (1). Phosphotungstic acid fraction; ↑ dose ≡ 1.6 g. yeast.
 „ (2). Alcoholic fraction; ↑ dose ≡ 1.76 g. yeast.
 „ (3). Platinum precipitate fraction; ↑ dose ≡ 2.37 g. yeast.
 „ (4). Platinum precipitate fraction; ↑ dose ≡ 3.16 g. yeast.

Stage 7. Fractionation by platinic chloride. The alcoholic extract was concentrated in a vacuum desiccator to a small bulk, and, after the removal of a light pale yellow precipitate which had separated, the filtrate (30 cc.) was treated with 6.5 cc. (excess) of a 5 % solution of platinic chloride in absolute alcohol. It rapidly deposited a micro-crystalline orange-yellow precipitate, which was left in the refrigerator overnight. As previous experiments showed that decomposition of the platinum precipitate by hydrogen sulphide frequently results in a considerable loss of activity¹, the platinum precipitate, after being filtered off and washed with a little absolute alcohol, was decomposed by means of potassium chloride by the method of Ewins [1914]. The platinum filtrate was also similarly decomposed. The platinum precipitate was active in doses containing 0.075–0.10 mg. total solids, equivalent to 2.37–3.16 g. yeast (Fig. 4). The platinum filtrate was entirely inactive in doses equivalent to 5 g. yeast. A mixture of half the effective dose of the platinum precipitate and a dose of the platinum filtrate, equivalent to 2.5 g. of original yeast, was almost entirely inactive.

Curative tests with 5 birds showed that the average day-dose of the platinum precipitate fraction contained 0.047 mg. total solids.

¹ In two instances about 80 % of the activity disappeared on treatment of the platinum precipitate with hydrogen sulphide. Such a large loss under the same treatment was not found to occur with wheat germ fractions [cf. Guha and Drummond, 1929], though the KCl method is undoubtedly a better one for such purposes.

Stage 8. Fractionation by gold chloride. The solution obtained by the decomposition of the platinum precipitate was evaporated to dryness in a vacuum desiccator. The residue was extracted with 13 cc. of hot absolute alcohol, filtered from a small amount of insoluble residue, and treated with 2 cc. of a 4 % solution of gold chloride in absolute alcohol. As no precipitate was formed, the solution was concentrated to a very small volume (0.5 cc.) in a desiccator, and then filtered from a very minute amount of an orange-yellow semi-crystalline precipitate. The gold precipitate, thus formed, was much more soluble in alcohol than in water.

The gold precipitate was dissolved in 10 cc. of 90 % alcohol and shaken up with precipitated metallic silver, according to the method of Dudley [1929], to precipitate the gold. The gold filtrate was also similarly freed from gold. The above operation, however, resulted in a great loss of activity. The fraction from the gold precipitate produced only slight growth in doses containing 0.054 mg. total solids, equivalent to 6 g. of the original yeast, while the gold filtrate was inactive in the same dose but produced slight growth in doses, containing 0.2 mg. total solids, equivalent to 11.2 g. of yeast. A mixture of 0.0357 mg. of the gold precipitate and 0.1 mg. of the gold filtrate resulted also in very slight growth (Fig. 5).

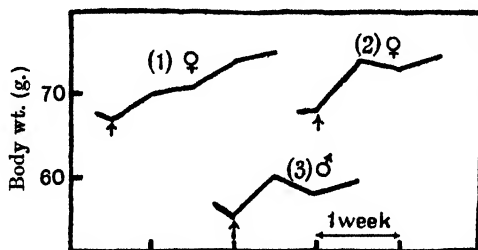


Fig. 5.

- Curve (1). Gold precipitate fraction; ↑ dose = 0.054 mg. total solids.
 „ (2). Gold filtrate fraction; ↑ dose = 0.2 mg. total solids.
 „ (3). ↑ dose = 0.0357 mg. of the gold precipitate fraction *plus* 0.1 mg. of the gold filtrate fraction.

Curiously, though the rat-growth tests gave such unsatisfactory results, the pigeon-curative tests indicated that there was quite considerable activity in both the gold precipitate and filtrate fractions, the former having twice

Gold precipitate fraction			Gold filtrate fraction			Gold precipitate fraction (g.p.) <i>plus</i> Gold filtrate fraction (g.f.)		
Pigeon no.	Dose injected (mg.)	Days of protection	Pigeon no.	Dose injected (mg.)	Days of protection	Dose injected (mg.)		Days of protection
						g.p.	g.f.	
1	0.05	3	1	0.1	8	1	0.05 + 0.05	8
2	0.05	2	2	0.15	2	2	0.05 + 0.05	5
3	0.05	6	3	0.25	8	3	0.05 + 0.05	9
4	0.05	7	4	0.3	16	Average day-dose = 0.014 mg.		
Average day-dose = 0.012 mg.			Average day-dose = 0.024 mg.					

the activity of the latter. A combination of the two fractions did not show any markedly enhanced effect. These results are shown in the above tables.

These curious results are discussed later.

Notes on the course of fractionation.

(1) The extraction of the brewer's yeast, if carried out simply by boiling with water, also gives satisfactory results and the fractionation can be followed to the platinum stage as described above.

(2) The precipitation by lead acetate at Stage 2 does not effect any considerable concentration because, while some inactive material is removed, a certain amount of loss occurs due, probably, to adsorption. But unless this preliminary purification is carried out, the subsequent fractionation by fuller's earth at Stage 3 does not appear to be so satisfactory. Adsorption by charcoal also ceases to be selective and occurs imperfectly at p_H 3, 4, 5 and 6, unless the lead stage is introduced. Kinnersley and Peters [1928] insert the lead stage before adsorption with charcoal.

(3) Levene and Van der Hoeven [1924] considered that barium ions precipitate a portion at least of the vitamin. Seidell [1926] was, therefore, led to the use of sodium hydroxide instead of saturated baryta for the elution of the vitamin from the "activated" fuller's earth. As it was not desired to introduce soluble inorganic material unnecessarily, the question was re-investigated. Under identical conditions elutions by equal volumes of $N/8$ NaOH and saturated baryta are equally effective, though one extraction by $N/8$ NaOH is more effective than one by $N/8$ Ba(OH)₂. There does not therefore appear to be any ground for assuming that the vitamin forms an insoluble complex with barium ions.

(4) It has been found that kieselguhr at p_H 4.5, can also adsorb the vitamin which can be eluted by saturated baryta. The adsorption by kieselguhr is, however, far less efficient than that by fuller's earth, as only about one-third of the vitamin is adsorbed by the former.

Vitamin B₁ and secretin.

Three different preparations of vitamin B₁, one obtained at Stage 1 by aqueous-alcoholic extraction, another at Stage 2 after lead fractionation and a third prepared by extracting with water, adsorbing with norite charcoal and fractionating with phosphotungstic acid, which were all highly active, were examined for their secretagogue activity. The tests were kindly carried out by Dr Brocklehurst and Mr Cunningham on amytal-anaesthetised cats. The third concentrated preparation did not show any appreciable effect at all, while the first two impure preparations had such a great depressor effect that in one case the animal was almost instantly killed. The nature of this depressor principle is not understood.

These observations confirm the earlier experiments of Anrep and Drummond [1921], as opposed to those of Uhlmann [1918] and of Voegtlin and Myers [1919].

Vitamin B₁ and "bios."

In confirmation of the earlier observations with wheat germ preparations [Guha and Drummond, 1929] it has been found that the "bios" activity of the yeast fraction obtained at Stage 5 is negligible, though this latter fraction was potent for rats in a daily dose of 0.3 mg. It is, however, of interest to point out in this connection that Williams and Roehm [1930] consider that while vitamin B₁ cannot stimulate the growth of yeast by itself, it can do so in conjunction with a material obtained from yeast extracts, which is not adsorbed by fuller's earth. Jansen and Donath's crystals are stated to exhibit this effect in minute amounts. In fact, from a parallel study of the growth of different varieties of yeast, Williams and Bradway [1931] have come to the conclusion that while "bios," as required by Wildier's strain of yeast and presumably by Narayanan's [1930], has not been fractionated, the strain used by the Toronto workers requires multiple factors, of which apparently inositol is one. Williams and Bradway consider that there are four "bios" factors and there might be some relation between vitamin B₁ and one of these. The writer's observations do not cover this point, as the tests were carried out with the same strain of yeast as was used by Narayanan.

Vitamin B₁ and co-zymase.

Kinnersley and Peters [1928] and Euler and Myrbäck [1929] consider that vitamin B₁ and co-zymase are not identical. As the most potent co-zymase preparations have been found to give a strong Molisch reaction and to contain phosphorus [Euler and Myrbäck, 1930] these properties have been investigated in some of our preparations.

2 cc. of the fraction obtained from the gold precipitate, which contained at least 10 rat day-doses, gave a negative reaction. Of the fractions obtained by the electrodialysis of a purified vitamin B₁ concentrate at p_H 8.5 (Birch and Guha, unpublished results) the fraction obtained in the cathode compartment, which contained at least 40 % of the activity, gave a negative Molisch reaction, while the fraction of the middle compartment gave a positive one. 2 cc. of a preparation obtained from rice polishings, potent for rats in 0.02 cc., kindly supplied by Prof. J. C. Drummond, which had been concentrated up to the platinum stage, still gave a positive reaction. This indicates, incidentally, that certain neutral impurities may be got rid of more readily by electrodialysis under suitable conditions than by metallic fractionation. Unlike co-zymase preparations, however, Drummond's concentrate was practically phosphorus-free. All these observations indicate that vitamin B₁ is probably not very closely related in structure to co-zymase. Also co-zymase is more readily destroyed by heat under certain conditions than vitamin B₁ and is susceptible to the action of nucleotidases. Vitamin B₁ does not appear to be very sensitive to the action of enzymes.

The absorption spectra of certain vitamin concentrates.

It was considered of interest to investigate whether vitamin B₁ concentrates show any selective absorption, which may be attributed to the vitamin. A search of the literature showed that Damianovich [1922] had observed an absorption band between 248 and 260 $\mu\mu$ given by an active brewer's yeast extract, which appeared to indicate pyrimidine compounds. Mukherji [1929] has recently observed that the crystals, which he obtained from rice polishings by Jansen and Donath's procedure, exhibited marked absorption at 390–330 $\mu\mu$.

The absorption spectra of (1) the cathode fraction obtained from yeast by fractionating up to Stage 5 and then electrolysing at p_H 8.5, and of (2) Drummond's rice-polishings concentrate, were examined with the ultra-violet spectrophotometer of the Hilger pattern (Fig. 6). The yeast concentrate

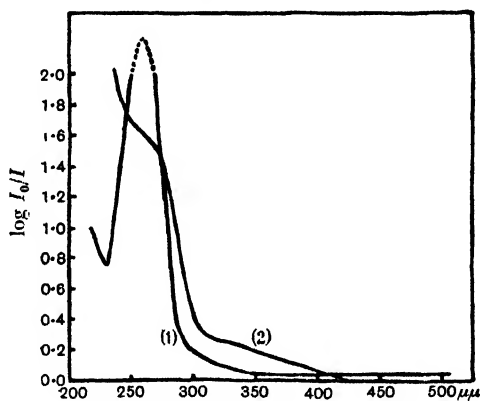


Fig. 6.

- Curve (1). The absorption spectrum of the electrolysised yeast fraction. 100 cc. of the solution contained 40 rat day-doses. Thickness of cell = 2 cm.
Curve (2). The absorption spectrum of Drummond's rice-polishings concentrate. 100 cc. of the solution contained 500 rat day-doses. Thickness of cell = 2 cm.

showed a very strong absorption in the region of 260 $\mu\mu$, which apparently accords with the observation of Damianovich. The same type of band, however, was observed by Dixon and Keilin to be given by a protein precipitate obtained by treatment with sulphur dioxide in course of fractionation for cytochrome from baker's yeast (private communication). This substance was kindly provided by Dr Keilin and, on being tested, it was found to be devoid of vitamin B₁ potency even in 10 mg. doses. This band is therefore not specific for B₁. The nature of the substance causing this absorption is unknown. Drummond's concentrate, which was much more potent than the electrolysised fraction, did not show this band (it was examined in three dilutions). The band observed by Mukherji was not shown markedly by either of the fractions. It is unlikely that a study of the absorption spectra of vitamin B concentrates will yield any significant results. It should be pointed out that

if vitamin B₁ has the glyoxaline structure as suggested by Jansen and Donath [1926], it may not have any selective absorption, as is indicated by the observations of Marchlewski and Nowotnowna [1925-6] on histidine.

Certain other properties of the vitamin concentrates.

1. *Behaviour towards flavianic acid.*

The fuller's earth extract did not give any appreciable precipitate with flavianic acid even after standing for 5 hours.

2. *Optical activity.*

The phosphotungstic fraction was examined for optical activity with reference to the mercury green line and at 18°, but there was no perceptible rotation, while the fraction obtained at the lead stage showed only a slight activity.

3. *Ninhydrin and Adamkiewicz-Hopkins reactions.*

The preparation obtained after fractionation with phosphotungstic acid and alcohol gave a negative ninhydrin reaction. A fraction purified by electro-dialysis at p_H 8.5 gave a very feeble Adamkiewicz-Hopkins reaction, while Drummond's fraction obtained from rice polishings, did not give this reaction.

4. *Pauly reaction.*

The Pauly reaction given by Jansen and Donath's crystals was one of the grounds on which they ascribed a glyoxaline structure to the vitamin [Jansen and Donath, 1926]. This reaction is given rather more strongly by the potent vitamin B₁ concentrates from yeast obtained by Kinnersley and Peters, and both reactions are less intense than that given by histidine [Jansen *et al.*, 1930]. We had observed a strong Pauly reaction with our most potent concentrate from wheat germ [Guha and Drummond, 1929]. The preparations obtained at the platinum stage by the method described in this paper have been found to give a very poor Pauly reaction compared with histidine. This reaction as given by these different concentrates cannot, therefore, be considered as trustworthy evidence about the chemical nature of vitamin B₁.

DISCUSSION.

The fractionation described in this paper gives very regular results, if followed in detail. It is difficult to understand the phenomenon observed at the stage of gold fractionation. The rat-growth tests failed to agree well with the curative tests on pigeons. One explanation might be that there is a difference between the rat-growth factor (or factors) and the pigeon curative factor (or factors). On the other hand, it is necessary to point out that the particular pigeon tests with the gold fractions showed very large individual variations and it is probable that the results are untrustworthy. This error could only be partially eliminated if an unusually large number of pigeons

were used. The results obtained with the rats are much more regular and these indicate that there is an enormous loss of vitamin B₁ at the gold fractionation stage, and the supplementary effect of the fractions obtained from the gold precipitate and filtrate, if any, cannot be expected to be observed under the circumstances. The question therefore must be considered still unsettled.

About the actual chemical nature of vitamin B₁ nothing can yet be said with confidence. Jansen and Donath's crystals have now been stated to contain vitamin B₄ [Jansen *et al.*, 1930]. Williams, Waterman and Gurin [1930] in a recent investigation could not obtain the pure crystals, while Mukherji [1929] states that by following Jansen and Donath's procedure in the main and working with rice polishings he has obtained crystals identical with those described by Jansen and Donath. It is not clear, however, that the crystals were pure. The tests, which were carried out on rice-birds (*Munia maja*) showed that 30 mg. of the crystals per day were effective for protection from "polyneuritis." Lower doses do not appear to have been tried. The Pauly reaction given by different vitamin B₁ concentrates from rice polishings and yeast cannot yet safely be attributed to the vitamin *per se*.

SUMMARY.

1. Adenine, guanine, uracil, *dl*-thyroxine, histamine and adrenaline cannot replace vitamin B₁ in the diet of rats.
2. A fairly standardised method of concentration of vitamin B₁ is described by which, starting from brewer's yeast, a preparation can be obtained at the platinum stage, which promotes good growth in rats in a daily dose of 0.075–0.1 mg. when supplemented by vitamin B₂. Its pigeon-curative day-dose is of the order of 0.047 mg.
3. No clear-cut answer has been obtained to the question whether fractionation by gold chloride would provide any evidence for the splitting of vitamin B₁. The rat-growth tests and pigeon-curative tests of these fractions show serious discrepancies.
4. Vitamin B₁ does not appear to be closely chemically related to cozymase. Its relation to secretin and "bios" is discussed.
5. Some vitamin B₁ concentrates are optically inactive and give negative ninhydrin and Adamkiewicz-Hopkins reactions. The Pauly reaction given by the purer yeast concentrates is comparatively weak. Vitamin B₁ is not apparently precipitated by flavianic acid from a fairly concentrated preparation.
6. The absorption spectra of certain concentrates are described.

I wish to express my thanks to Sir F. G. Hopkins and Prof. J. C. Drummond for their stimulating interest, and also to Dr M. Dixon for his interest and generous help in the work on the absorption spectra of the concentrates.

REFERENCES.

- Anrep and Drummond (1921). *J. Physiol.* **54**, 349.
 Carter, Kinnersley and Peters (1930, 1). *Biochem. J.* **24**, 1832.
 ——— (1930, 2). *Biochem. J.* **24**, 1844.
 Damianovich (1922). *Anales Asoc. Quim. Argentina*, **10**, 209.
 Dudley (1929). *Biochem. J.* **23**, 1064.
 Euler and Myrbäck (1929). *Svensk. Kem. Tid.* **41**, 209.
 ——— (1930). *Z. physiol. Chem.* **190**, 93.
 Ewins (1914). *Biochem. J.* **8**, 44.
 Guha (1929). *Chem. Ind.* **48**; *Proc. Biochem. Soc.* 1248.
 ——— and Drummond (1929). *Biochem. J.* **23**, 880.
 Jansen and Donath (1926). *Proc. K. Akad. Wetensch., Amsterdam*, **29**, 1390.
 ——— Kinnersley, Peters and Reader (1930). *Biochem. J.* **24**, 1824.
 Kinnersley and Peters (1928). *Biochem. J.* **22**, 419.
 ——— (1930). *Biochem. J.* **24**, 1856.
 ——— and Reader (1930). *Biochem. J.* **24**, 1820.
 Levene and Van der Hoeven (1924). *J. Biol. Chem.* **61**, 429.
 Marchlewski and Nowotnowna (1925-6). *Acad. Polonaise Bull.* **43**, 153.
 Mukherji (1929). *Indian Med. Gaz.* **64**, 443.
 Narayanan (1930). *Biochem. J.* **24**, 6.
 Pirie (1930). *Biochem. J.* **24**, 51.
 Quinn, Whalen and Hartley (1930). *J. Nutr.* **3**, 257.
 Reader (1930). *Biochem. J.* **24**, 1827.
 Seidell (1926). *J. Biol. Chem.* **67**, 593.
 Smith (1930). *U.S. Publ. Health Rep.* **45**, 116.
 Uhlmann (1918). *Z. Biol.* **68**, 419.
 Voegtlin and Myers (1919). *J. Pharm. exp. Therap.* **13**, 301.
 Williams and Bradway (1931). *J. Amer. Chem. Soc.* **53**, 783.
 ——— and Roehm (1930). *J. Biol. Chem.* **87**, 581.
 ——— and Waterman (1928). *J. Biol. Chem.* **78**, 311.
 ——— and Gurin (1930). *J. Biol. Chem.* **87**, 559.

CV. INVESTIGATIONS ON VITAMIN B₂.

- I. THE SOURCES OF VITAMIN B₂.
- II. THE STABILITY OF VITAMIN B₂.
- III. THE CHEMISTRY OF VITAMIN B₂.

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SINCE the recognition of the presence of at least two factors, B₁ and B₂, in the dietary complex known as "vitamin B," little work has been published on the nature and behaviour of vitamin B₂. The present investigation was prompted by the need for fuller knowledge of this subject. Preliminary reports of some of the results have appeared elsewhere [Guha, 1931, 2, 3].

BIOLOGICAL TECHNIQUE.

Young rats of about 50 g. in weight were kept in separate cages with screened bottoms and were fed on the basal diet described in a preceding paper [Guha, 1931, 1]. Each animal received separately in a dish a daily dose of 2 drops of cod-liver oil and of a preparation of vitamin B₁ from the beginning of the experimental feeding period. The B₁-preparation was obtained from brewer's top yeast by extracting with aqueous alcohol and then fractionating with neutral lead acetate [Guha, 1931, 1]. In some experiments the filtrate from the lead precipitate was further purified by adsorption on fuller's earth by the method described before. In others, however, the lead filtrate itself was used as the source of vitamin B₁ and was fed in a daily dose of 0.5 c.c., equivalent to 1-1.5 g. of the fresh yeast. This preparation contained a small amount of vitamin B₂, but as both positive and negative controls were kept, the results were checked throughout. Rats on the above dietary began to decline in weight in 2-4 weeks from the commencement of the experiment, according to the reserve of vitamin B₂ in their bodies. Test fractions were fed at this stage and if growth occurred at a rate of 10-12 g. per week for 2-3 weeks, the fractions were considered to be active. Fig. 1 shows that the B₁-preparation (lead filtrate) cannot by itself produce growth in 0.5 cc., but can produce slow growth in 3-4 times that dose. If, however, 0.5 cc. of the B₁-preparation is supplemented by 1 cc. of a 50 % solution of alkalised marmite [Guha, 1931, 1] as a source of vitamin B₂, good growth is obtained, whereas the alkalised marmite by itself cannot sustain growth even in a daily dose of 4 cc.

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The B_1 -preparation made by means of fuller's earth is almost entirely devoid of vitamin B_2 and is probably to be preferred in all tests for vitamin B_2 . The lead filtrate, however, is likely to contain other factors [Reader, 1930], which might be an advantage.

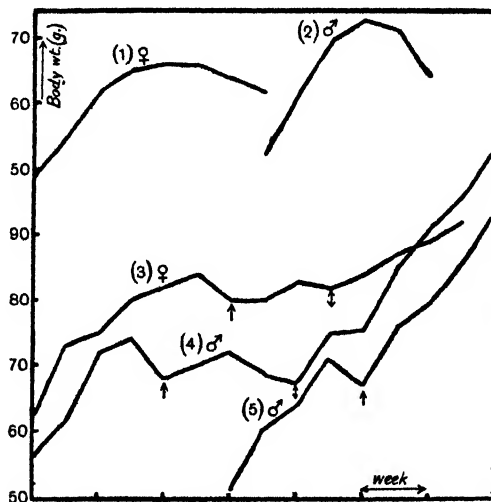


Fig. 1.

- Curve (1). 0.5 cc. of vitamin B_1 preparation (lead filtrate) from the beginning of experiment.
 Curve (2). 1 cc. of vitamin B_2 preparation (alkalised marmite) from the beginning of experiment.
 Curve (3). 0.5 cc. of lead filtrate from the beginning. At \uparrow given 1 cc. of lead filtrate. At \downarrow given 3.0 cc. of lead filtrate.
 Curve (4). 1 cc. of alkalised marmite from the beginning. At \uparrow 4 cc. of the same. At \downarrow 1 cc. of alkalised marmite plus 0.5 cc. of lead filtrate.
 Curve (5). 1 cc. of alkalised marmite from the beginning. At \uparrow 0.5 c.c. of lead filtrate added.

No case of refection was noticed in these experiments though uncooked rice starch was used in the basal diet. It is our custom, however, to check the results by observing whether the withdrawal of an active supplement is followed by a decline in weight.

I. THE SOURCES OF VITAMIN B_2 .

It was early realised that the recognition of the dual nature of "vitamin B" required a revision of the "vitamin B" values of foodstuffs, especially where these values were obtained by growth tests on rats. Aykroyd and Roscoe [1929] and Roscoe [1930] have recently assayed vitamin B_2 in certain materials.

The present estimation of vitamin B_2 in certain raw materials was carried out with the purpose of finding out which of these would be most useful for the extraction of the vitamin. Aqueous extracts of brewer's top yeast, baker's yeast, ox-liver, beef-muscle, commercial liver extract (Eli Lilly, No. 343)¹, and milk powder were examined.

¹ I am indebted to Dr C. Elvehjem for the commercial liver extract used in this research.

Brewer's yeast, baker's yeast, ox-liver and beef-muscle were extracted under identical conditions, the liver and muscle being finely minced before extraction. 300 g. of each of them were stirred gradually into 480 cc. of boiling distilled water and the boiling continued for 3 minutes.

These were filtered under suction and washed each with 100 cc. water. The muscle gave a practically colourless solution, while the liver yielded an opalescent brownish liquid with a faint green fluorescence.

Eli Lilly's commercial liver extract was fed both in the solid state and also as an aqueous extract which was prepared by stirring 50 g. of the liver extract with 200 cc. of cold water. This was filtered under suction from a small amount of insoluble residue which was washed with a little water. The deep brown-red filtrate was made up to 250 cc. to make a 20 % solution. The residue was apparently a remnant of the liver protein. It dissolved in *N*/10 NaOH and was precipitated by the careful addition of *N*/10 HCl at *p*_H about 4.6, which is close to the isoelectric point of the liver protein. Both the residue and the aqueous extract were tested for activity.

"Glaxo" milk powder, which was prepared from English winter milk, was fed both in the solid state and in an aqueous medium.

The results of these tests are shown in Figs. 2 and 3. It will be observed

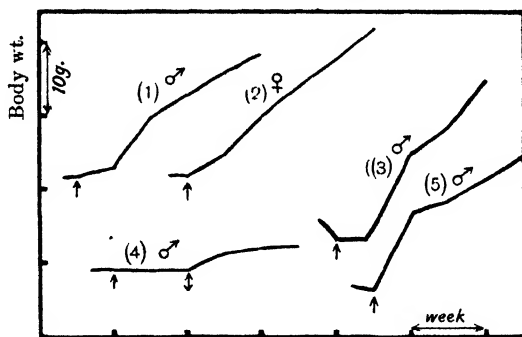


Fig. 2.

- Curve (1). ↑ dose ≡ 1.4 g. fresh brewer's yeast.
 Curve (2). ↑ dose ≡ 0.7 g. fresh baker's yeast.
 Curve (3). ↑ dose ≡ 0.94 g. fresh baker's yeast.
 Curve (4). ↑ dose ≡ 0.9 g. fresh beef muscle; ↓ dose ≡ 1.8 g. fresh beef muscle.
 Curve (5). ↑ dose = 0.4 g. milk powder.

that the extract of baker's yeast is markedly richer in vitamin B₂ than that of the brewer's yeast, the former giving as good growth in a dose corresponding to 0.7-0.9 g. of fresh yeast as the latter in a dose equivalent to 1.4 g. Some hope was entertained about milk powder as a raw material for the fractionation of vitamin B₂, as the major constituents of this material are known. But it proved to be active only in a dose as large as 0.4 g. The muscle extract was comparatively inactive, which is rather surprising, considering that, according to Goldberger, lean meat is a rich source of the antipellagra factor. Vitamin B₂ may, however, be present in muscle in a combination from which

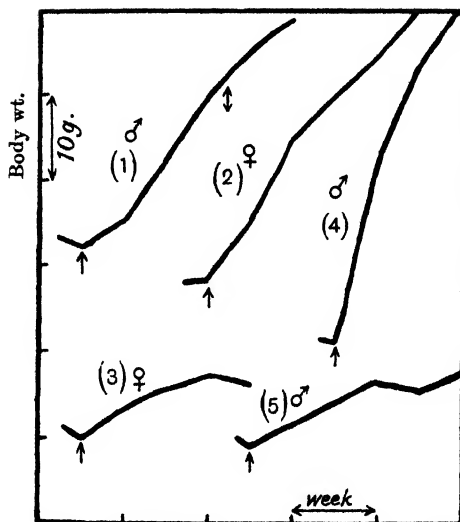


Fig. 3.

- Curve (1). Aqueous extract of fresh ox-liver; ↑ dose \equiv 0.3 g. of fresh liver (containing 6.0 mg. organic material); ↓ dose \equiv 0.15 g. of fresh liver.
 Curve (2). A cold aqueous extract of Eli Lilly's commercial liver concentrate; ↑ dose \equiv 50 mg. of the liver concentrate.
 Curve (3). The insoluble residue obtained after a cold aqueous extraction of the commercial liver concentrate; ↑ dose \equiv 0.25 g. of the concentrate.
 Curve (4). Commercial liver concentrate; ↑ dose \equiv 0.15 g.
 Curve (5). Shows the vitamin B₁ potency of the cold aqueous extract of commercial liver concentrate; ↑ dose \equiv 0.12 g. of the concentrate.

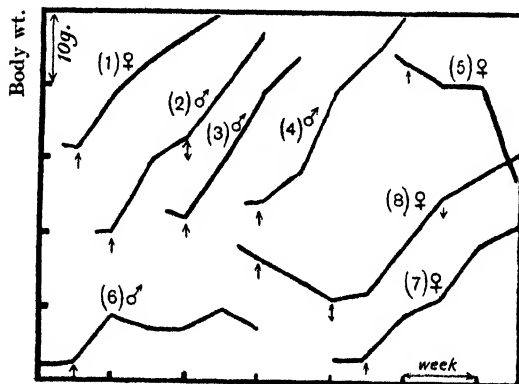


Fig. 4.

- Curve (1). Aqueous extract of commercial liver concentrate, autoclaved at p_H 9.0 for $\frac{1}{2}$ hour; ↑ dose \equiv 60 mg. of the concentrate.
 Curve (2). Same extract, autoclaved at p_H 9.0 for 3 hours; ↑ dose \equiv 90 mg. of the concentrate; ↓ dose \equiv 60 mg. of the concentrate.
 Curve (3). Same extract, autoclaved at p_H 7.4 for 3 hours; ↑ dose \equiv 60 mg. of the concentrate.
 Curve (4). Same extract, autoclaved at p_H 6.2 for 3 hours; ↑ dose \equiv 60 mg. of the concentrate.
 Curve (5). Vitamin B₁ potency of the same extract, autoclaved at p_H 9.0 for $\frac{1}{2}$ hour; ↑ dose \equiv 180 mg. of the concentrate.
 Curve (6). Brewer's yeast extract, autoclaved at p_H 9.0 for 3 hours; ↑ dose \equiv 4.6 g. of fresh yeast.
 Curve (7). Same extract, autoclaved at p_H 6.25 for 3 hours; ↑ dose \equiv 1.7 g. of fresh yeast.
 Curve (8). Aqueous extract of fresh ox-liver, autoclaved at p_H 9 for 3 hours; ↑ dose \equiv 0.3 g. of fresh liver; ↓ dose \equiv 0.9 g. of liver; ↓ dose \equiv 0.6 g. of liver.

it is difficult to separate it by boiling water. The muscle residue was not tested for its activity. The aqueous extract of fresh ox-liver was extremely potent, being active in a daily dose containing less than 6 mg. total solids, which were equivalent to about 0.3 g. of the fresh liver. This extract should serve as a promising source of vitamin B₂ for fractionation and other purposes.

The cold aqueous extract of the commercial liver concentrate was highly active in a daily dose of 40–60 mg. The residue was practically inactive. This 20 % aqueous extract was used for the chemical study described in this paper. This material has some advantages over yeast, which has been so far generally used as a source of vitamin B₂. It is more potent, and it can be readily prepared from a stock of the solid liver concentrate whenever required. This ensures a greater uniformity of results. It is, moreover, comparatively poor in vitamin B₁, which is an advantage for certain purposes. The 20 % aqueous extract keeps well in the refrigerator.

II. THE STABILITY OF VITAMIN B₂.

There has lately been a serious disagreement among different workers about the stability of vitamin B₂, especially to heat in an alkaline medium. Goldberger's work had shown that the factor concerned in the prevention of pellagra was relatively thermostable [Goldberger *et al.*, 1926]. In fact, the relative thermostability of vitamin B₂ has so far been the main factor in its differentiation from the more heat labile vitamin B₁. Other workers have also found that the vitamin B₂ potency of the commercial yeast extract, marmite, is not very seriously reduced by autoclaving for 3 hours at 120° at an alkaline reaction [Hassan and Drummond, 1927; Guha and Drummond, 1929; Narayanan and Drummond, 1930]. That a preparation of alkaline-autoclaved marmite can supplement a concentrated preparation of vitamin B₁ for the growth of vitamin B-deficient rats has been confirmed by us in hundreds of cases. On the other hand, other workers have stated that autoclaving in an alkaline medium destroys vitamin B₂ in brewer's yeast and its extracts, made by boiling with 0.01 % acetic acid, almost entirely [Williams, Waterman and Gurin, 1929; Chick and Roscoe, 1930].

It appeared to the writer at the outset that these discrepancies about the stability of vitamin B₂ might be due to the different behaviour of vitamin B₂ when present in association with different substances. That such is the case with vitamin B₁ is now well established [Kinnersley and Peters, 1928; Guha and Drummond, 1929]. An investigation was, therefore, instituted to find out the vitamin B₂ potency of some of the extracts, described in Part I, after autoclaving under identical conditions.

Aqueous extracts of brewer's top yeast, fresh ox-liver and the commercial liver concentrate were made as described and their p_H adjusted with $N/2$ NaOH. The treatment undergone by the extracts and the results on testing are summarised in the following table (see also Fig. 4).

Nature of material				Period of autoclaving (hours)	Temp. of autoclaving	Initial p_H	Final p_H	Per-centage of inactivation (roughly)
Extract of fresh ox-liver		3	124–125°	9.0	6.2	75
Extract of commercial liver concentrate				3	124–125°	9.0	6.1	0
"	"			$\frac{1}{2}$	124–125°	9.0	6.5	0
"	"			3	124–125°	7.4	4.87	0
"	"			3	124–125°	6.2	4.65	0
Brewer's yeast extract	3	124–125°	9.0	7.5	90
"	3	124–125°	6.25	5.62	0

Thus the curious fact is revealed that while vitamin B_2 in commercial liver extract and also in marmite [Hassan and Drummond, 1927] possesses a very high degree of stability, vitamin B_2 in the extracts made by boiling liver and fresh yeast with water is very markedly unstable to alkaline autoclaving. As the 20 % solution of the commercial liver extract contained about 5 times more solids than the brewer's yeast extract, the possibility could not be excluded that the greater dilution of the latter was helping its inactivation. The solution of the liver concentrate was therefore diluted 5 times and autoclaved at p_H 9 for 3 hours at 124–125°, when the p_H changed to 7.6. There was very slight inactivation, if any at all. It is probable that the stability of vitamin B_2 in a given preparation is due to the presence of some kind of protective material in it. Two possibilities present themselves: (1) that the commercial liver extract and marmite contain something which is added to them by the manufacturers and which has a protective action on vitamin B_2 , (2) that there is a natural protective agent present in fresh liver and yeast, which is removed by the method of boiling with water, but which is not removed entirely in the manufacturing methods employed. The writer is inclined to think that the latter is the more probable explanation. The protective action of certain colloids, for instance of gelatin and saponin on gold sol, is well known.

Half an hour's autoclaving of the 20 % aqueous solution of the commercial liver extract at p_H 9 at 124–125°, while it did not affect vitamin B_2 , destroyed vitamin B_1 almost entirely. Such a preparation would, therefore, appear very suitable for purposes where vitamin B_2 , free from vitamin B_1 , is needed. As this preparation contains much less solid matter and is apparently more palatable to the rats, it is to be preferred to autoclaved marmite.

III. THE CHEMISTRY OF VITAMIN B_2 .

Few facts have been recorded in the literature on the concentration and chemistry of vitamin B_2 . Chick and Roscoe [1929] observed that it was precipitated to a large extent from an aqueous yeast extract by neutral lead acetate. Narayanan and Drummond [1930] obtained a concentrate from yeast by successive fractionations with lead acetate and alcohol. Levene [1930] reports that he has obtained a preparation of vitamin B_2 , which is potent for the growth of young rats in a daily dose of 0.7 mg.

The 20 % aqueous solution of Eli Lilly's liver extract, described in Part I, or the solid extract itself was used as the material for the present study. The potency of a fraction was, as usual, established by testing on at least two animals, frequently more. In no case was a serious discrepancy observed between the responses of two rats to a given fraction.

1. *Treatment with alcohol.* On treatment of 5 g. of the solid liver extract with 50 cc. of 58 % alcohol, most of the material dissolved giving a red solution. The residue (0.37 g.) was dissolved in water with a few drops of *N*/10 NaOH. About 65 % of the activity passed into the alcoholic extract, the rest being left in the residue (Fig. 5). There was no marked loss of activity

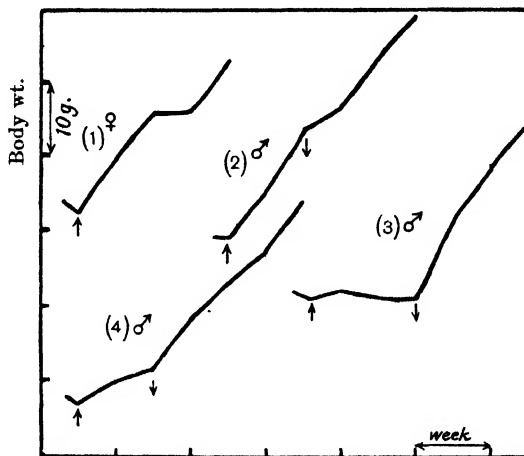


Fig. 5.

Curve (1). Fraction insoluble in 58 % alcohol; ↑ dose \equiv 0.125 g. of the liver concentrate.

Curve (2). Fraction soluble in 58 % alcohol; ↑ dose \equiv 0.125 g. of the liver concentrate; ↓ dose \equiv 0.100 g. of the concentrate.

Curve (3). ↑ Picric acid precipitate, dose \equiv 0.2 g. of the liver concentrate; ↓ picric acid filtrate, dose \equiv 50 mg. of the liver concentrate.

Curve (4). Acetone-precipitated fraction; ↑ dose \equiv 75 mg. of the liver concentrate; ↓ dose \equiv 0.125 g. of concentrate (containing about 8 mg. organic material).

[cf. Chick and Copping, 1930]. But the treatment with 58 % alcohol does not apparently cause a clear-cut separation of vitamin B₂ from inactive material.

2. *Treatment with picric acid.* 100 cc. of the 20 % aqueous solution of the liver concentrate were treated with a moderate excess of a hot saturated aqueous solution of picric acid. After cooling, the clear red supernatant fluid was removed from the somewhat gummy precipitate, both were decomposed by hydrochloric acid and the picric acid was repeatedly extracted with ether and then with benzene. The material obtained from the picric acid precipitate was entirely inactive, while that from the picric acid filtrate retained practically all the activity (Fig. 5). This experiment shows that the vitamin is almost insoluble in ether and benzene. Most of the vitamin B₂ potency of the original liver extract was also obtained in the picric acid filtrate.

Fractionation with acetone. The picric acid filtrate which had a p_H of about 1, was treated with 6 times its volume of acetone, when an oily precipitate came down. After leaving the mixture in the refrigerator for 2 days in a corked flask, the clear reddish-orange supernatant liquid was poured off from the gummy brown residue. The residue, after being dried with a stream of air, was found to contain a little more than 50 % of the activity (Fig. 5). The mother-liquor was brought to p_H 6 and again treated with an excess of acetone, when a second oily precipitate separated, but both this precipitate and the filtrate were inactive.

The active fraction obtained by the first precipitation with acetone gave a negative purine test, a doubtful Millon's test and positive biuret and Adamkiewicz-Hopkins reactions. It also gave a very marked Molisch reaction, a fairly positive reaction with Tollen's reagent and a negative one with Bial's. It reduced Benedict's reagent and gave a positive sulphur reaction on prolonged boiling with lead acetate and concentrated sodium hydroxide.

The active picric acid filtrate fraction does not give a precipitate with flavianic acid even after standing for 3-4 hours.

3. *Action of benzoyl chloride.* 30 cc. of the 20 % solution of the liver concentrate were shaken up with 4 cc. of benzoyl chloride and excess of 20 % NaOH in the usual way. A considerable quantity of a yellow precipitate

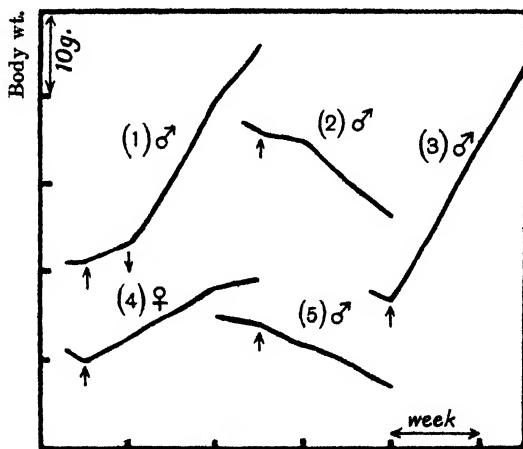


Fig. 6.

- Curve (1). Filtrate from the precipitate obtained with benzoyl chloride; \uparrow dose \equiv 90 mg. of the liver concentrate; \downarrow dose \equiv 180 mg. of the concentrate.
 Curve (2). The precipitate formed with benzoyl chloride; \uparrow dose \equiv 0.6 g. of the concentrate.
 Curve (3). Filtrate from the precipitate obtained with nitrous acid; \uparrow dose \equiv 60 mg. of concentrate.
 Curve (4). Filtrate from the precipitate obtained with phosphotungstic acid; \uparrow dose \equiv 0.3 g. of the concentrate.
 Curve (5). Phosphotungstic acid precipitate fraction; \uparrow dose \equiv 0.3 g. of the concentrate.

came down, which was removed and dried in a desiccator (weight = 3.5 g.). The filtrate was treated with hydrochloric acid and the benzoic acid removed with ether. This filtrate retained about one-third of the activity, while the precipitate was inactive (Fig. 6).

4. *Action of nitrous acid.* Contrary to Levene [1928], Chick [1929] and Narayanan and Drummond [1930] reported that vitamin B₂ of yeast extracts was stable to nitrous acid. Vitamin B₂ of the 20 % solution of the liver concentrate was also found to be neither precipitated nor inactivated by nitrous acid.

30 cc. of the 20 % solution, acidified with hydrochloric acid, were treated in the usual way with a sufficient volume of a concentrated solution of sodium nitrite, as indicated by starch iodide paper. This was warmed at 60° for half an hour and the nitrous fumes expelled by boiling for 2 minutes in an open beaker. The precipitate, which had come down by treatment with nitrous acid, was inactive, while the filtrate retained the entire activity (Fig. 6).

5. *Reaction with phosphotungstic acid.* 25 cc. of the solution of liver extract were made 5 % acid with concentrated H₂SO₄ and treated with 45 cc. of saturated phosphotungstic acid in 5 % H₂SO₄. After standing overnight, the precipitate, which had removed most of the pigment from the solution, was filtered off. Both the precipitate and filtrate were freed from phosphotungstic acid by means of baryta, barium being finally removed with sulphuric acid. The precipitate fraction was entirely inactive, while the material obtained from the phosphotungstic filtrate was only very slightly active (Fig. 6). A combination of the precipitate and filtrate fractions did not give any more satisfactory results. It is highly probable that the vitamin was removed by adsorption on the surface of barium phosphotungstate and barium sulphate.

6. *Treatment with baryta.* 50 cc. of the 20 % solution were treated with 60 cc. of saturated baryta and left overnight. The resulting precipitate was removed and freed from barium by sulphuric acid (fraction a). The filtrate and washings from the barium precipitate were treated with an equal volume of alcohol and allowed to stand for 3 hours. The precipitate which had separated was filtered off and freed similarly from barium (fraction b). The filtrate from the second barium precipitate, which was still highly pigmented, was acidified with sulphuric acid and concentrated under reduced pressure (fraction c). Fractions (a) and (b) were inactive, while fraction (c) retained a little more than 30 % of the original activity (Fig. 7).

7. *Reaction with lead acetate.* 50 cc. of the aqueous liver extract, which was normally at p_H 4.6, on treatment with 20 cc. saturated neutral lead acetate (a slight excess) deposited a small quantity of precipitate. After standing overnight, this was removed and both precipitate and filtrate were decomposed by means of sulphuric acid. Vitamin B₂ was found to be partially precipitated by this procedure (Fig. 7).

Since treatment with lead acetate at p_H 4.6 did not cause a complete separation of vitamin B₂, the experiment was repeated at p_H 7 with no better results. The lead precipitates in either case were practically free from vitamin B₁. The lead precipitate fraction can thus be used as a source of vitamin B₂, free from vitamin B₁, but the method does not appear to have any great advantage over that described in Part II.

The lead precipitate fraction obtained at p_H 4.6 may be further fractionated by bringing it to p_H 6.8 and then treating it with 6 volumes of acetone. The active material is again precipitated as in the case of the picric acid precipitate.

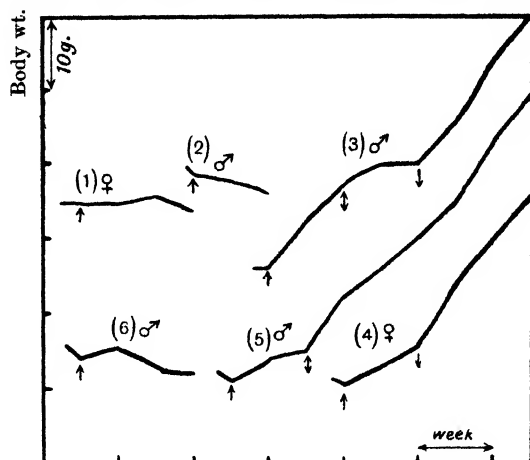


Fig. 7.

- Curve (1). Fractionation with baryta; fraction *a*; ↑ dose \equiv 0.2 g. of liver concentrate.
 Curve (2). Fractionation with baryta; fraction *b*; ↑ dose \equiv 0.2 g. of liver concentrate.
 Curve (3). Fractionation with baryta; fraction *c*; ↑ dose \equiv 0.2 g. of concentrate; ↓ dose \equiv 0.1 g. of concentrate; ↓ dose \equiv 0.15 g. of concentrate.
 Curve (4). Lead filtrate; ↑ dose \equiv 0.1 g. of concentrate; ↓ dose \equiv 0.2 g. of concentrate.
 Curve (5). Lead precipitate; ↑ dose \equiv 0.1 g. of concentrate; ↓ dose \equiv 0.15 g. of concentrate.
 Curve (6). Shows the vitamin B_1 potency of the lead precipitate fraction; ↑ dose \equiv 0.2 g. of concentrate.

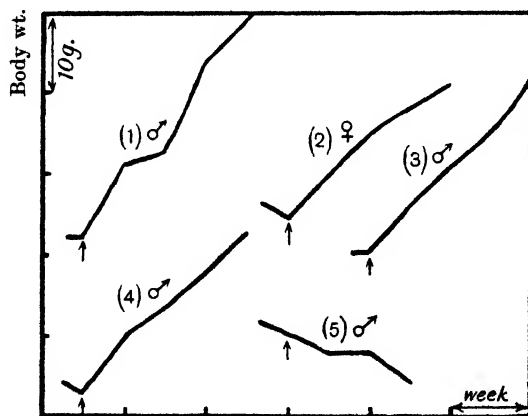


Fig. 8.

- Curve (1). Silver nitrate precipitate fraction; ↑ dose \equiv 0.1 g. of liver concentrate.
 Curve (2). Silver nitrate filtrate fraction; ↑ dose \equiv 0.3 g. of liver concentrate.
 Curve (3). Filtrate from precipitate with litharge; ↑ dose \equiv 75 mg. of concentrate.
 Curve (4). Non-esterified fraction; ↑ dose \equiv 0.15 g. of concentrate.
 Curve (5). Esterified fraction; ↑ dose \equiv 0.4 g. of concentrate.

8. *Reaction with silver nitrate.* 20 cc. of the aqueous liver extract were treated with 13 cc. of a 25 % solution of silver nitrate, the mixture left over-

night in the dark and the precipitate filtered off. Silver was removed from both the precipitate and filtrate by treatment with HCl and subsequent centrifugation (Fig. 8). The precipitate fraction was much more potent than the filtrate fraction. It appears that this method causes a more effective separation of vitamin B₂ from inactive material than does the reaction with lead acetate.

A portion of the silver precipitate fraction was brought to p_H 9 and autoclaved for 3 hours at 124–125°. The p_H changed to 8.0 and the preparation was found to be at least half inactivated. This shows that purification by certain methods may make the vitamin less stable to alkaline autoclaving, probably by removing some protective material (*cf.* Part II).

9. *Treatment with litharge.* 25 cc. of the aqueous liver extract were shaken up with 3 g. (excess) of litharge. After filtration the residue and the filtrate were freed from lead by sulphuric acid. Practically all the activity was present in the filtrate (Fig. 8).

10. *Esterification with ethyl alcohol.* 4 g. of the solid liver extract were covered with 120 cc. absolute alcohol and treated with dry hydrogen chloride in the usual way for 5 hours. In the last hour the temperature of the reaction vessel was maintained at 50–60°. Nearly all the material dissolved giving a dark brown-red solution. The contents of the flask were poured into a beaker and chilled to 0°. Two volumes of water were slowly added and then silver carbonate and silver oxide. When the p_H of the solution was still below 1, the precipitate, which appeared to consist wholly of silver chloride, was filtered off under suction and well washed and the washings added to the filtrate. The dark red solution was again brought to 0° and then very slowly treated with 20 % sodium hydroxide with constant stirring until the p_H was about 9.5 (the temperature should not rise above 5°). The solution which now measured 525 cc., was immediately extracted 4 times with a total volume of about 750 cc. ether. The ethereal layer was pale yellow, most of the pigment being in the aqueous layer. The ethereal layer was washed with very dilute hydrochloric acid and then with water, which removed some more of the colouring matter. After evaporating the ether by means of an electric fan at 30° the residue was taken up in water (fraction *a*). The aqueous layer was acidified with HCl, concentrated under reduced pressure, and filtered from the precipitated sodium chloride. As it was, however, still extremely saline and could not be fed to the animals, it was saturated with hydrogen chloride at 0°. After removing the large amount of precipitated sodium chloride the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in water, brought to p_H about 6 and fed to the animals (fraction *b*). Fraction *a* was inactive, while about 30–40 % of the original activity was present in fraction *b* (Fig. 8). The recovery appears to be good enough to warrant the conclusion that the vitamin is not esterifiable under the aforesaid conditions of experiment.

11. *Adsorption on charcoal.* It has been the experience of other workers

[Salmon, Guerrant and Hays, 1928; Narayanan and Drummond, 1930] that vitamin B₂ of yeast extracts is tenaciously adsorbed by certain substances, from which it is difficult to remove by elution. Such has also been the experience of the writer in using charcoal for the adsorption of vitamin B₂ from the liver concentrate.

100 cc. of the aqueous liver extract (p_H circa 4.6), were shaken up with 6.7 g. of norite charcoal for 5-7 minutes. After filtration, the charcoal weighed 12-15 g., and was potent in doses corresponding to 80-100 mg. of the original liver extract. The filtrate was practically inactive (Fig. 9).

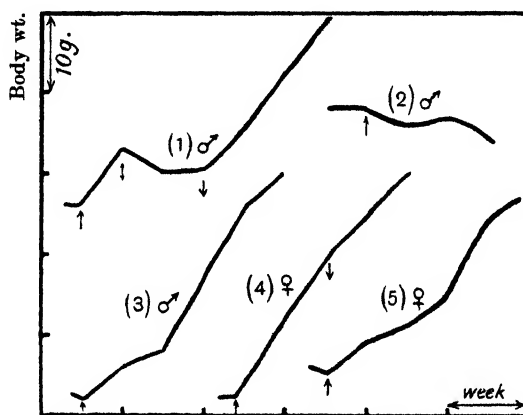


Fig. 9.

Curve (1). Activated charcoal; ↑ dose ≡ 0.1 g. of liver concentrate; ↓ dose ≡ 75 mg. of concentrate; ↓ dose ≡ 90 mg. of concentrate.

Curve (2). Charcoal filtrate; ↑ dose ≡ 0.18 g. of concentrate.

Curve (3). SO₂-treated preparation; ↑ dose ≡ 60 mg. of the concentrate.

Curve (4). O₃-treated preparation; ↑ dose ≡ 80 mg. of concentrate; ↓ dose ≡ 60 mg. of concentrate.

Curve (5). H₂O₂-treated preparation; ↑ dose ≡ 80 mg. of concentrate.

5 g. portions of this activated charcoal were extracted with 50 cc. each of 0.1 N HCl, 0.1 N NaOH, 30 % ethyl alcohol, containing 0.5 cc. concentrated hydrochloric acid and 45 % alcohol, containing 0.5 cc. of 20 % NaOH. The extractions were carried out by heating under reflux on a water-bath for 1 hour. The extract in every case was practically inactive. The charcoal residues were in some cases active, but the activity had been materially reduced. 5 g. of the activated charcoal were also extracted successively with 200 cc., 200 cc. and 100 cc. of 30 % propyl alcohol, the flask being heated each time at 75° for 10 minutes. The extract was slightly active, and the residue practically inactive. Another portion of 5 g. of the activated charcoal was extracted twice with 125 cc. of 0.2 % saponin ("pure," B.D.H.) in the cold, and the mixture centrifuged, but the centrifugate was inactive.

12. *Stability to sulphur dioxide.* Sulphur dioxide was passed into 20 cc. of the aqueous liver extract for 4 hours. The excess of SO₂ was boiled off in an open beaker. The vitamin is completely stable to this treatment (Fig. 9).

13. *Stability to hydrogen peroxide.* 20 cc. of the aqueous extract were treated with 5 cc. of Merck's perhydrol, heated for 1½ hours at 70° and then evaporated to dryness in an open basin in a water-bath. The residue was dissolved in water. No very marked inactivation occurred (Fig. 9).

14. *Stability to ozone.* 20 cc. of the aqueous extract were treated with a rapid stream of ozonised oxygen for 70 minutes. The colour of the solution gradually faded to a lemon-yellow. This was concentrated under reduced pressure. The vitamin was fairly stable to such treatment (Fig. 9).

15. *Action of trypsin.* 80 cc. of the aqueous liver extract were brought to p_H 8. 1 g. powdered trypsin (B.D.H.), which had been found in this laboratory to be active, was added. The mixture was incubated after the addition of a little toluene for 7 days at 37°. Another 80 cc. of the aqueous liver extract was similarly incubated without trypsin as a control. The p_H of the digested solution had changed to 7.2 in 7 days. Both the solutions were acidified with hydrochloric acid, concentrated *in vacuo* to remove toluene and tested. Both of them were active. The trypsin powder itself exhibited no activity even in relatively large doses.

16. *The behaviour of vitamin B₂ on electrodialysis.* Preliminary experiments carried out with Mr T. W. Birch on the electrodialysis of vitamin B₂ of the aqueous liver extract, indicate that the vitamin is probably a neutral substance. It remains concentrated in the middle compartment under the conditions tried, while small quantities appear to be present in both the anode and cathode compartments, this being apparently due to diffusion through the parchment membrane.

DISCUSSION.

The behaviour of vitamin B₂ of the liver extract to basic precipitants like phosphotungstic acid, benzoyl chloride, picric acid, *etc.* indicates that the vitamin is probably not a basic substance. On the other hand, the fact that esterification of the extract leaves a fair amount of the vitamin in the non-esterified fraction, while the esterified fraction is inactive, argues against the acid nature of the vitamin. The occurrence of both vitamin B₂ and the factor specific for pernicious anaemia in large quantities in liver raises the question of a relation between the two substances. The fact that a lowering of the erythrocyte count has been observed in vitamin B₂ deficiency [Sure, Kik and Smith, 1931] also lends point to this question. The general chemical behaviour of the vitamin, as shown in this paper, is however in contrast to that of the factor for pernicious anaemia, which has recently been stated by Dakin, West and Howe [1930] to be a compound of β -hydroxyglutamic acid and *l*- γ -hydroxyproline.

The idea gained from general chemical evidence that vitamin B₂ is probably a neutral substance is apparently corroborated by the experiments on the electrodialysis of the vitamin. The precipitation of a large portion of the

activity with lead acetate and silver nitrate is probably due to the adsorption of the vitamin by the precipitates formed.

CERTAIN OTHER OBSERVATIONS.

Among the rats, which were kept for periods of 10 weeks or so on a vitamin B₂-deficient diet in course of the experiments described in this paper, with only occasional administrations of small doses of vitamin B₂, a considerable proportion developed a curious form of depilation. The fur fell off not in patches but uniformly in such a manner as to give the rats a "close-cropped" appearance [Guha, 1931, 2]. Only in a very small proportion of cases did lesions appear near the mouth and on the paws. The hair began to grow within 2 or 3 days of the administration of the liver concentrate as a source of vitamin B₂ and within 7 or 8 days assumed the normal "lying-down" appearance. This condition of depilation has occurred fairly regularly when the deprivation of vitamin B₂ is not complete but sufficiently severe to cause the weight of the animals to remain between 60 and 80 g. over a period of 10–12 weeks [cf. Leader, 1930; Sure and Smith, 1931].

The above symptoms did not improve nor did growth resume on feeding lactalbumin, haemoglobin or haemin¹ in daily doses of 1 g., 0.5 g. and 25 mg. respectively [cf. Kollath, 1929; Bliss, 1930; Bliss and Thomason, 1931].

SUMMARY.

1. The values of the following as sources of vitamin B₂ have been investigated—milk powder and aqueous extracts of brewer's yeast, baker's yeast, fresh ox-liver, beef-muscle and Eli Lilly's liver concentrate No. 343. The fresh ox-liver extract is apparently the most potent of these. An aqueous extract of the liver concentrate is also very potent, being effective in a daily dose of 40–60 mg. for the growth of young rats. The advantages of this liver concentrate over yeast as a source of vitamin B₂ are pointed out.

2. The stability of vitamin B₂ preparations obtained from different sources towards heat and alkali shows curious discrepancies. Thus the vitamin B₂ in aqueous extracts of yeast and fresh ox-liver is much less stable than that in marmite and in the commercial liver concentrate. It is probable that the stability of certain preparations is connected with the presence of some kind of protective material in them. An aqueous extract of the liver concentrate autoclaved at p_H 9 for half an hour at 124–125° provides an excellent source of vitamin B₂ free from vitamin B₁.

3. A chemical study of vitamin B₂ in a cold aqueous extract of the liver concentrate has been made. Picric acid, benzoyl chloride, phosphotungstic acid and flavianic acid do not precipitate the vitamin. Nitrous acid neither precipitates nor inactivates it. Lead acetate and silver nitrate precipitate it partially. Esterification leaves about 40 % of the vitamin in the non-esterified

¹ I am indebted to Mr Meldrum for this preparation of haemin.

fraction, the esterified fraction being inactive. It is not attacked by trypsin. Norite charcoal adsorbs the vitamin at the normal p_H (4.6) of the aqueous extract of the liver concentrate. It was not possible to elute it effectively by aqueous alcohol, 30 % propyl alcohol or by dilute saponin. On the basis of the present evidence it may be provisionally concluded that the vitamin is probably not a base, acid or peptide, but a neutral substance. Preliminary experiments on the electrodialysis of the vitamin support this tentative conclusion. The partial precipitation by lead acetate and silver nitrate is probably due to the adsorption of the vitamin on the precipitates formed. The vitamin is stable to sulphur dioxide, hydrogen peroxide and ozone.

4. Some of the rats which were maintained at a weight between 60 and 80 g. over periods of 12 weeks or so, and were not undergoing a drastic deprivation of vitamin B₂, developed a curious form of depilation, which was cured by administration of the liver extract.

5. Haemin, haemoglobin and lactalbumin could not ameliorate the above symptoms or produce growth in absence of vitamin B₂.

I desire to express my gratitude to Sir F. G. Hopkins for his interest in this work and to thank Mr A. Ward and Miss Ruby Leader for their skilful assistance with the animals.

REFERENCES.

- Aykroyd and Roscoe (1929). *Biochem. J.* **23**, 483.
Bliss (1930). *Science*, **72**, 578.
—— and Thomason (1930–31). *Proc. Soc. Exp. Biol. Med.* **28**, 636.
Chick (1929). *Biochem. J.* **23**, 514.
—— and Copping (1930). *Biochem. J.* **24**, 1744.
—— and Roscoe (1929). *Biochem. J.* **23**, 504.
—— (1930). *Biochem. J.* **24**, 105.
Dakin, West and Howe (1930–31). *Proc. Soc. Exp. Biol. Med.* **28**, 2.
Goldberger, Wheeler, Lillie and Ropers (1926). *U.S.A. Publ. Health Rep.* **41**, 297.
Guha (1931, 1). *Biochem. J.* **25**, 931.
—— (1931, 2). *Lancet*, **i**, 864.
—— (1931, 3). *Nature*, **127**, 594.
—— and Drummond (1929). *Biochem. J.* **23**, 880.
Hassan and Drummond (1927). *Biochem. J.* **21**, 653.
Kinnersley and Peters (1928). *Biochem. J.* **22**, 419.
Kollath (1929). *Arch. exp. Path. Pharm.* **142**, 86.
Leader (1930). *Biochem. J.* **24**, 1172.
Levene (1928). *J. Biol. Chem.* **79**, 465.
—— (1930). *Science*, **71**, 668.
Narayanan and Drummond (1930). *Biochem. J.* **24**, 19.
Reader (1930). *Biochem. J.* **24**, 77.
Roscoe (1930). *Biochem. J.* **24**, 1754.
Salmon, Guerrant and Hays (1928). *J. Biol. Chem.* **80**, 91.
Sure, Kik and Smith (1930–31). *Proc. Soc. Exp. Biol. Med.* **28**, 498.
—— and Smith (1930–31). *Proc. Soc. Exp. Biol. Med.* **28**, 442.
Williams, Waterman and Gurin (1929). *J. Biol. Chem.* **83**, 321.

CVI. OBSERVATIONS ON CERTAIN FACTORS NECESSARY FOR THE NORMAL NUTRITION OF THE RAT.

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INTRODUCTION.

IN course of attempts made to isolate vitamin B₁ [Guha and Drummond, 1929], it was observed early in 1928 that if in these experiments the animals were kept in a state of more or less stunted growth by alternate administration and withdrawal of vitamin B₁ concentrates over a period of 10–12 weeks, the subsequent prolonged administration of vitamin B₁ concentrates of known potency (as determined by pigeon curative tests and growth tests on younger rats) did not permit continued good growth in these animals. Growth was not effectively restored by increasing the doses of vitamin B₁ and B₂ concentrates (the latter being supplied as alkaline-autoclaved marmite). But if they were replaced by relatively small quantities of whole brewer's yeast, dried at 35°, or of marmite, the animals responded with much more continued growth [Guha and Drummond, 1929, p. 896, footnote]. Even so, growth ceased after some time at a point well below the normal. At this stage, milk was tried, partly empirically and partly with the classical experiments of Hopkins [1912] in mind, with distinctly beneficial results. These preliminary observations suggested the possibility that in yeast and in milk we might be dealing with factors other than the known vitamins. As, however, other explanations might well be given for the observed results, these preliminary experiments were followed up and an investigation carried out during the last 3 years with reference to the following points.

- (1) Can the observed effect be ascribed to a better protein in the supplement or to increased amounts of the known vitamins A, B₁, B₂, C, D or E?
- (2) Is the increased growth associated with increased food intake? If so, would it be possible to have increased growth with restricted food intake?
- (3) To extract, if possible, the new factor (or factors) from natural sources and define its chemical character.

In this investigation over one hundred rats were used, some of which were kept under observation for experimental periods exceeding 35 weeks. Extensive food-intake records were kept. No attempt was made to find if the

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absence of the new factor (or factors) would affect breeding and lactation. It should be emphasised that entirely regular results were not obtained, but the effect of the factors concerned was well outside the limits of individual biological variations and was sufficiently regular to warrant the conclusions reached. It may also be pointed out that part of this work was carried out at University College, London, and part at the Biochemical Laboratory, Cambridge, with animals reared on different diets. The maximum attainable weights of the stock rats also differ. Still, the results obtained at the two laboratories agree fairly, and the effects of the absence or administration of the new factors are similar. Unfortunately, not many experiments could be carried out on the extraction of the factors concerned and no suitable method has yet been devised for this purpose. A preliminary report of this work has appeared elsewhere [Guha, 1930].

Since 1926 a number of papers have appeared bearing more or less directly on the same subject [Osborne and Mendel, 1926; Palmer and Kennedy, 1927; Evans and Burr, 1927, 1; Hunt, 1928; Reader, 1928, 1929, 1930; Coward, Key and Morgan, 1929; Mason, 1929; Chick and Copping, 1930]. This work is discussed later in relation to the present investigation.

EXPERIMENTAL.

The rats were kept in separate cages with screened bottoms, and supplements were given, wherever necessary, in separate dishes and, in general, the procedure described by Guha and Drummond [1929] was followed. The rats used at University College, London, were raised on a diet of the following composition:

					%
Maize meal	63
Whole wheat	20
"Casein" (B.D.H. "light white")	9
Dried yeast powder		4
Calcium carbonate		3
Common salt	1

Some sheep-liver was supplied once a week. These rats were normally capable of attaining a maximum weight of 300 g. for males and about 210 g. for females, which usually took about 30–34 weeks. The rats at the Cambridge laboratory were bred on a dietary of brown bread, fresh milk and whole wheat, and usually reached a maximum weight of 350–360 g. for males and about 250 g. for females. The animals used at University College and those at Cambridge are designated as "A Class" and "B Class" rats respectively. They were all of the black and white strain. In the following experiments 2 drops of cod-liver oil were given daily to each rat in a dish, unless otherwise mentioned.

A. *Experiments showing the effect of vitamin B₁ and B₂ preparations, yeast, marmite and milk, on rats, whose growth had been artificially stunted over a considerable period.*

Fig. 1 illustrates cases of A Class rats, in which, owing to the alternate administration and withdrawal of vitamin B₁ preparations, the rats were kept at a subnormal weight over considerable periods. These rats were throughout on a basal diet composed of 75 % rice starch, 21 % "light white casein"

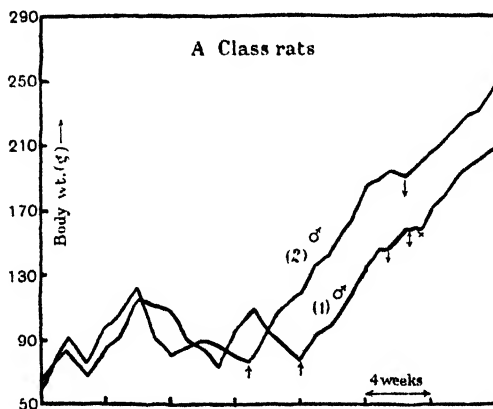


Fig. 1.

Curve (1). ↑ 0.5 cc. of a vitamin B₁ concentrate from wheat germ *plus* 1 cc. of 50 % alkalisied marmite; ↓ the dose of each doubled; ⚡ the dose of each trebled; × 0.5 g. dried brewer's yeast daily.

Curve (2). ↑ 0.5 g. fresh marmite daily; ↓ 15 cc. of milk in addition.

(B.D.H.) and 4 % salt mixture (McCollum). In addition, they received daily doses of 2 drops of cod-liver oil of proved potency and 1.0 cc. of a 50 % solution of alkaline-autoclaved marmite [Guha and Drummond, 1929]. After the expiration of 10–12 weeks from the beginning of experimental feeding, the administration of a vitamin B₁ concentrate, in the form of a fuller's earth extract obtained from wheat germ [Guha and Drummond, 1929] or of a charcoal concentrate obtained from brewer's yeast by the acid-alcohol method of Kinnersley and Peters [1928], could not maintain continued growth. Doubling or trebling the dose of the vitamin B₁ concentrate and of the alkalisied marmite produced only a temporary response. The increased effect of brewer's yeast (dried in the laboratory at 35° by means of an electric fan), and of fresh untreated marmite over vitamin B₁ and B₂ preparations, and of fresh pasteurised milk over marmite was quite noticeable. That the vitamin B₁ concentrates used in these experiments were highly potent in small doses (0.5 cc.) was shown by growth tests on younger rats. The activity of the fuller's earth extract was also checked by curative tests on pigeons, the day-dose being found to be of the order of 0.1–0.15 cc. That autoclaved marmite affords a very potent preparation of vitamin B₂ has also been demonstrated

[Hassan and Drummond, 1927] and has been confirmed by the writer on numerous occasions [Guha, 1931].

B. Experiments demonstrating the increased effect of dried brewer's yeast over that produced by relatively large doses of vitamin B₁ and B₂ preparations on normal growing rats.

While the effects of yeast and milk, described in Section A, were suggestive and were actually first observed as a side-issue in our work on the fractionation of vitamin B₁, it was obvious that the fact that these observations were made under conditions in which growth was artificially inhibited over a fairly long period would complicate the conclusions. Experiments were, therefore, carried out with young growing A Class rats, which were given vitamin B₁ and B₂ preparations of known potency from the start of the experimental feeding period. In addition to vitamins A and D, some of these animals were also provided with a vitamin E concentrate from wheat germ oil. Here also growth usually fell off after about 9 or 10 weeks and while increased doses of vitamins B₁ and B₂ temporarily stimulated growth, it could be effectively restored by a relatively small dose of dried brewer's yeast (Fig. 2). This did

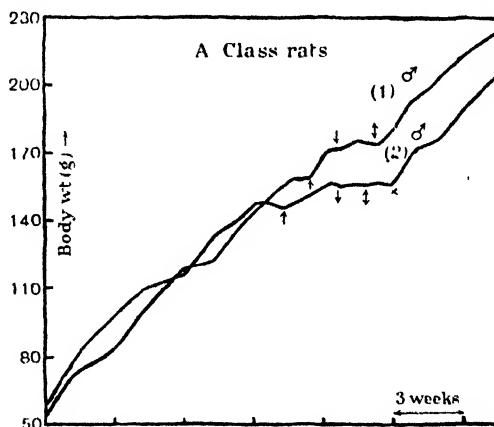


Fig. 2.

Curve (1). 0.5 cc. of fuller's earth preparation from wheat germ plus 1 cc. of 50 % alkalised marmite from the beginning of experiment; ↑ dose of each supplement doubled; ↓ dose of each quadrupled; ♂ 0.5 g. dried brewer's yeast daily.

Curve (2). 0.5 cc. of fuller's earth concentrate from wheat germ plus 1 cc. of 50 % alkalised marmite from the beginning of experiment; ↑ dose of each supplement doubled; ↓ 1 g. yeast residue in addition; ♂ 0.2 g. yeast ash in addition; × the above supplements replaced by 0.5 g. dried brewer's yeast.

not occur on feeding the ash of yeast or the residue of yeast obtained after extraction with boiling water. The supposition, that in these experiments yeast exerted its effect by means of its protein content, is rendered improbable by the fact that the same basal diet as described in Section A was used, which contained commercial "light white casein," a comparatively uninjured protein. The observations of Osborne and Mendel [1925], that rats required larger

amounts of "vitamin B" at later stages of the growing period, might receive a new interpretation in the light of these experiments.

C. *The effect of milk on the growth of rats receiving vitamin B₁ and B₂ preparations.*

It was found that when rats were exhibiting subnormal growth after being on vitamin B₁ and B₂ preparations for 9 or 10 weeks, 8–15 cc. of fresh unskimmed milk would produce a resumption in growth (Fig. 3). This appears

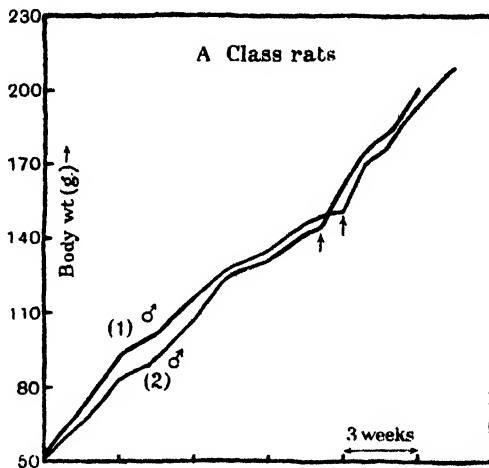


Fig. 3.

Curve (1). 0.5 cc. of fuller's earth preparation from wheat germ *plus* 2 cc. of 50 % alkalised marmite from the beginning of experiment; ↑ 8 cc. of unskimmed milk.

Curve (2). Same supplements as represented in Curve (1); ↑ 10 cc. of unskimmed milk.

to be far out of proportion to the amounts of vitamins B₁ and B₂ that milk contains, especially since, as has been shown in the previous sections, the effect of increased doses of vitamin B₁ and B₂ concentrates is not so marked. It would appear, therefore, that milk contains the additional factor present in yeast.

D. *Effect of milk on the growth of rats fed on a diet containing yeast.*

The observation that milk could supplement vitamins B₁ and B₂ led the writer to investigate what effect, if any, milk would have if the diet were already supplemented by yeast. Although in these early experiments, "light white casein" formed the protein of the basal diet, a distinct effect was observed if relatively large quantities of milk (15–25 cc.) were fed in addition. The experiments of Coward, Key and Morgan [1929] have since shown that "light white casein" contains some factor not present in "Glaxo vitamin-free casein." These findings had an obvious relation to those of the writer, and, therefore, in all subsequent experiments, "Glaxo vitamin-free casein" was substituted for the "light white" variety. It may, however, be stated here that "Glaxo casein," "light white casein" and purified caseinogen, as prepared by dis-

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solving commercial caseinogen in dilute sodium hydr by hydrochloric acid, show some difference in physical question could not be investigated in detail for lack of t that amongst these "light white casein" is most nearly p condition. It is probable that, for nutritional experiment of purified caseinogen, the original method of purification as adopted by Svedberg, Carpenter and Carpenter [1930] fo of pure caseinogen for molecular weight determinations, wou The use of ammonia instead of sodium hydroxide would proba risk of de-amination and racemisation.

In the following experiments B Class rats were used. The results were obtained with a basal diet composed of "Glaxo cas mercial sucrose, yeast and salt mixture (Osborne and Mendel), wit added fat. In subsequent experiments, however, the following diet w

				%
Commercial sucrose	60
"Glaxo vitamin-free casein"	21
Palm kernel oil	8
Dried yeast	7
Salt mixture (Osborne and Mendel)	4

Daily doses of 2 drops of cod-liver oil were given separately to each ra dish, according to the usual procedure. However, in some of these experi cod-liver oil (2.5 %) was incorporated in the diet with equally go In such a case, however, the diet should be made up every week. kernel oil, which in some experiments was replaced by an equal a olive oil, was added in order to provide some fat in the diet [Burr a. 1929]. On such a dietary the weight of the B Class male rats became or nearly so in the region of 200-220 g. after an experimental feeding of 10-12 weeks. The corresponding weight of the female rats was 150-1 When the rats attained this stage, fresh "certified" milk in varying c (10-25 cc.) had a pronounced effect, which could not be produced by (1 creasing the daily dose of cod-liver oil to 4 drops, (2) increased allowance vitamin B₁ and B₂ preparations and yeast, (3) orange juice (5 cc. daily, (4) olive oil (6 drops daily), (5) lactalbumin (1 g. daily) in the form of "albu lacticin," (6) the addition of the following amino-acids to the diet in the stated proportions:

					%
Cystine	1
<i>D</i> -Lysine dihydrochloride	0.5
Tryptophan	1
Histidine monohydrochloride	1
Arginine hydrate	1
Tyrosine	1

B. C. GUHA

oil (6 drops daily) obtained by extraction with cold
 a, heated at 130–170° for 3 hours—a treatment, which,

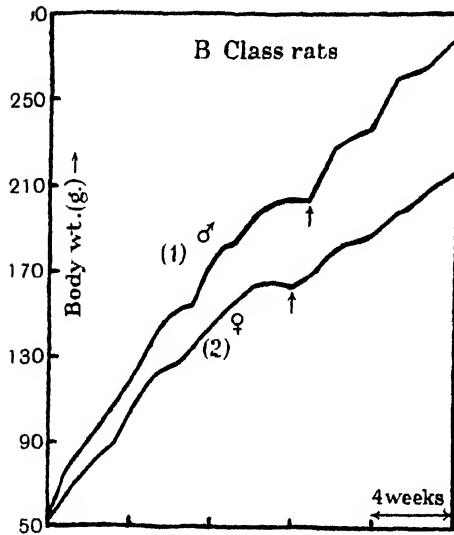


Fig. 4.

Curve (1). Basal diet as described in Section D; ↑ 15 cc. fresh certified milk daily.
 Curve (2). Basal diet as described in Section D; ↑ 15 cc. fresh certified milk daily.

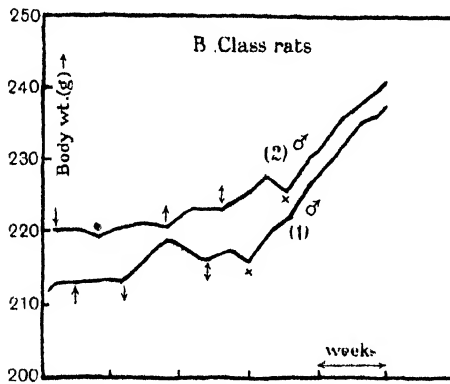


Fig. 5. Basal diet as described in Section D.

- Curve (1). Body weight steady after 11 weeks' feeding on basal diet; ↑ 4 drops of cod-liver oil added daily; ↓ 0.5 g. dried yeast added daily; ‡ 5 cc. of orange juice daily; × 15 cc. fresh certified milk daily.
- Curve (2). Body weight steady after 12 weeks' feeding on basal diet; ↓ 1 g. lactalbumin fed daily; ⊙ the mixture of essential amino-acids incorporated in the diet; ↑ 6 drops of olive oil daily; ‡ 15 % heated wheat germ incorporated in the diet; × 15 cc. fresh certified milk daily.

according to Evans and Burr [1927, 2], does not seriously affect vitamin E of the embryo—when introduced into the diet in a proportion of 15 % produced only a slight stimulation of growth. These experiments are illustrated

ACCESSORY FACTORS REQUIRED .

in Figs. 4 and 5. The effect of milk on the growth of males was 969 than on that of females. This difference in response between males and females has been noticed throughout this investigation and is correlated with the normally greater rate of growth of the male.

These experiments indicate that the factor present is not identified with vitamin A, C, D, E or any of the factors previously mentioned, also that the deficiency in growth in the absence of this substance is attributed to a deficiency of the so-called essential amino-acids. The possibility cannot, however, be excluded that some other substance, a peptide like that recently described by Dakin, West and Howe, or that the body may be unable to synthesise, may be the factor concerned.

E. *The sources of the new factor.*

The method by which this factor was assayed in different materials consisted in feeding these to the rats after their growth had stopped as a result of a feeding period of 10 weeks on the basal dietary described in the foregoing section. A weekly increase of 10 g. body weight for 3-4 weeks was considered as the standard of good growth. It has to be mentioned that milk does not produce sustained growth for a very long time and the requirements also vary considerably from animal to animal. The results obtained with some materials are given in the following table.

Nature of material	Dose	Result
Egg-yolk	1-1.5 g.	Good growth
Egg-white	2 g.	Fairly good growth
Pig-liver	2 g.	Slight growth
Sheep-liver	2 g.	Slight growth
Spinach (dried at 35°) ...	1-1.5 g.	Good growth
Grass (dried at 35°) ...	1-1.5 g.	Good growth
Alfalfa	1-1.5 g.	Fairly good growth
Certified Grade "A" milk	10-25 cc.	Good growth

The fact that milk contains considerable quantities of this factor suggests the possibility of its extraction from "Glaxo" milk powder. But neither the ethereal extract of this powder (15 drops of the melted fat) nor the powder itself in daily doses of 1 g. was capable of producing growth. It is possible that the substance was injured in the drying process. The cream of milk was not much more effective than milk itself.

Both spinach and grass (dried at 35° by means of an electric fan) were extracted with ether for 6 hours in a Soxhlet in an atmosphere of nitrogen. But the extracts were almost entirely inactive, while the residues produced only slight growth.

Finally, it has to be mentioned that both "light white casein" and milk show very varying degrees of activity. Considering the possibility of seasonal variations a scrutiny was made of the records, but the evidence obtained was not conclusive. However, it appears possible that if the experiments were carried out under more standardised conditions with a fixed source of milk

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...tion might be revealed, Spinach and grass appear active than milk, considering dry weights, and it seems that the ultimate source of the milk factor is the food of carotene, kindly provided by Dr Moore, in a daily dose of 10 mg. Likewise, chlorophyll, when incorporated in a portion of 0.15 %, proved entirely inactive.

The stability of the factor present in milk to heat.

The following table summarises the results, obtained very roughly.

Treatment	Time	Result
Heating at 12-13 lbs. pressure	40 mins.	Approx. half destroyed
	2½ hrs.	At least 75 % destroyed
Heating at 18 lbs. pressure	15 mins.	Less than half destroyed
	2½ hrs.	Wholly inactivated

G. The question of food consumption.

Very extensive food-intake records were kept, in some cases throughout the experimental period. These records show that, in many cases, when growth resumed after administration of milk, the food consumption did not rise

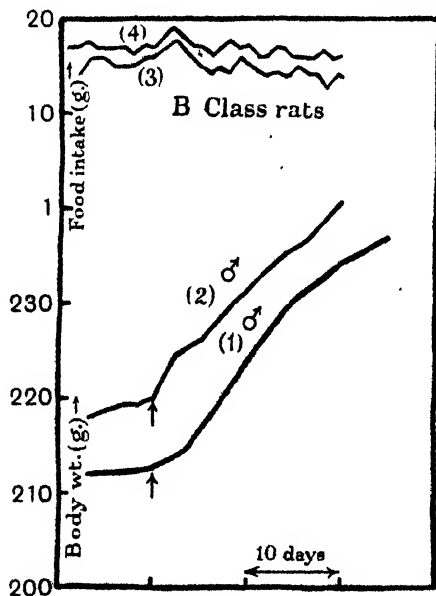


Fig. 6.

Curves (1) and (2). Basal diet as described in Section D; ↑ 15 cc. fresh certified milk daily.

Curve (3). Shows the food intake of the animal represented in Curve (1) before and after administration of milk.

Curve (4). Food-intake curve corresponding to Curve (2).

appreciably. Two such typical cases are shown in Fig. 6. Though in some other cases, the food intake rose, the fact, that it is possible to get resumption of

growth without greatly increased food consumption, suggests that this factor exerts its influence more directly on the assimilation of the food ingested than on appetite. If this is so, its effect should be reflected in a positive nitrogen balance.

DISCUSSION.

It is not necessary to examine in detail all the papers referred to before. It is significant that all those investigators, working, sometimes, under considerably different conditions, observed the inadequacy of the known factors for the normal nutrition of the rat. In some of this work, however, it is not clear whether the limiting factor might not have been "vitamin B," while in some other cases specially "purified" proteins were used and the possibility could not be excluded that the effect of the supplement given was merely a good protein effect. Preparations of the third factor in yeast, called vitamin B₄, have recently been obtained by Reader [1930]. The experiments described in this paper have a great resemblance to those of Coward, Key and Morgan [1929]. They were led from a study of the relative qualities of "light white casein" and "Glaxo casein" in the basal diets of rats for vitamin A and B tests to the discovery that the former variety of caseinogen contained some factor, in which the latter variety was deficient. They subsequently traced this substance in milk and in many other materials. The writer's observations, however, started with milk. It appears probable that we are dealing here with the same substance. It should be stated, however, that Coward has found ox-liver to be rich in the factor, while, according to my observations, pig-liver and sheep-liver appear to be deficient in it. Coward and her collaborators have also observed that it can be extracted from wheat embryo by 90 % alcohol and by ether. My preliminary attempts to extract it from spinach and grass have so far failed. Attention should also be called to the work of Mason [1929], who independently showed that lettuce provided some additional factor for growth and, by a critical analysis, proved that this factor was not identical with vitamin E, which is present in large quantities in lettuce [Evans and Bishop, 1923].

The experiments described in this paper appear to eliminate the effect of all the known vitamins. That this factor cannot be identical with vitamin E is also shown by the fact that wheat germ heated under conditions in which vitamin E is not destroyed [Evans and Burr, 1927, 2] loses its activity. That untreated wheat germ is rich in this factor has been shown by Coward. The protein effect would appear to be excluded by the negative results obtained with lactalbumin and with the essential amino-acids tested. The idea that it might be some inorganic material seems to be similarly excluded by the observation that the factor in milk can be inactivated by autoclaving. That the deficiency described here cannot be of the type encountered by Burr and Burr [1929] is shown by the practically negative effect of olive oil. However, no evidence about its chemical nature has yet been obtained.

It appears that the factor present in yeast may also be present in milk, but the factor present in milk is not present in appreciable quantities in yeast. The two factors, therefore, seem to be different. It is possible that the observation of earlier workers that "complete" synthetic rations produce good growth was due to an insufficiently long observational period. It is also felt that the growth curves would to a very considerable extent depend on the dietary on which the stock had been reared and also on the strain of rats used.

SUMMARY.

1. Accumulated evidence is presented to show that yeast contains a factor other than vitamin B₁ or B₂, and that milk contains a factor other than those present in yeast. Both the milk factor and the yeast factor are apparently required for the normal growth of rats. But even then entirely normal growth is not sustained.
2. The factor present in milk is different from other known vitamins, A, D, C or E.
3. The deficiency of the milk factor cannot be corrected by adding lactalbumin or the essential amino-acids to the diet.
4. Food-intake records show that increased growth on the administration of milk may occur without appreciably increased food intake.
5. The stability of the milk factor to heat has been investigated. The fact that it can be inactivated by autoclaving argues against the inorganic nature of this substance.
6. The factor present in milk is also present in egg-yolk, egg-white, spinach, grass and alfalfa, but much less so in pig- and sheep-liver.
7. Carotene or chlorophyll cannot replace the milk factor in the diet.
8. No satisfactory method has yet been devised for extracting the substance.
9. A method of assay of this factor is indicated.
10. Male rats show a much more marked response to this factor than females.
11. Milk and "light white casein" show appreciable variations in potency.

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REFERENCES.

- Burr and Burr (1929). *J. Biol. Chem.* **82**, 345.
Chick and Copping (1930). *Biochem. J.* **24**, 1764.
Coward, Key and Morgan (1929). *Biochem. J.* **23**, 695.
Dakin, West and Howe (1930-31). *Proc. Soc. Exp. Biol. Med.* **28**, 2.
Evans and Bishop (1923). *J. Metabol. Res.* **3**, 233.
—— and Burr (1927, 1). *Proc. Soc. Exp. Biol. Med.* **24**, 740.
—— ——— (1927, 2). The antisterility vitamin fat-soluble E. *Memoirs of the University of California*, vol. **8**.
Guth (1930). *Chem. Ind.* **49**, 850.
—— (1931). *Biochem. J.* **25**, 931.
—— and Drummond (1929). *Biochem. J.* **23**, 880.
Hassan and Drummond (1927). *Biochem. J.* **21**, 653.
Hopkins (1912). *J. Physiol.* **44**, 425.
Hunt (1928). *J. Biol. Chem.* **79**, 723.
Kinnorsley and Peters (1928). *Biochem. J.* **22**, 419.
Mason (1929). *J. Nutr.* **1**, 311.
Osborne and Mendel (1925). *J. Biol. Chem.* **63**, 233.
—— ——— (1926). *J. Biol. Chem.* **69**, 661.
Palmer and Kennedy (1927). *J. Biol. Chem.* **75**, 619.
Reader (1928). *Chem. Ind.* **47**, 1247.
—— (1929). *Biochem. J.* **23**, 689.
—— (1930). *Biochem. J.* **24**, 77.
Svedberg, Carpenter and Carpenter (1930). *J. Amer. Chem. Soc.* **52**, 241.

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